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NUEVOS BIOMARCADORES, ENFERMEDADES CARDIOVASCULARES Y ENFERMEDAD RENAL CRÓNICA DIABÉTICA

Tesis Doctoral

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Department of Medicine

NOVEL BIOMARKERS, CARDIOVASCULAR DISEASE AND DIABETIC CHRONIC KIDNEY DISEASE

Doctoral Thesis

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INFORME DEL DIRECTOR DE TESIS

Don Alberto Ortiz Arduán, Doctor en Medicina por la Universidad Autónoma de Madrid, y Doña Maria Dolores Sanchez Niño, Doctora por la Universidad Autónoma de Madrid,

CERTIFICAN

que Don Usama Abdalla Elewa, Licenciado en Medicina por la Universidad de Ain Shams - El Cairo - Egipto, ha realizado en el Servicio de Nefrología de la Fundación Jiménez Díaz, bajo su dirección el trabajo titulado ñNuevos Biomarcadores, Enfermedades Cardiovasculares Y Enfermedad Renal Crónica Diabéticaò que presenta como Tesis Doctoral para alcanzar el grado de Doctora por la Universidad Autónoma de Madrid.

Y para que conste, firmamos la presente en Madrid, a 9 de Mayo del 2014.

Dr. Alberto Ortiz Arduán **Dra. Maria Dolores Sanchez Niño**

Doctorando: Usama Abdalla Elewa

ñI have learned that success is to be measured not so much by the position that one has reached in life as by the obstacles which he has overcome while trying to succeedò

Booker T. Washington

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List of abbreviations

$1,25(OH)_2D$	1,25 Dihydroxivitamin D
25(OH)D	25 Hydroxyvitamin D
ABI	Ankle Brachial Index
ABIs	Ankle Brachial Indices
ACEI	Angiotensin Converting Enzyme Inhibitor
ADA	American Diabetes Association
ADAM	Asymmetrical Dimethylarginine
ADAM10	A Disintegrin and metalloproteinase domain-containing protein 10
ADVANCE	Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation
AER	Albumin Excretion Rate
AGEs	Advanced Glycosylation End-products
AIX	Augmentation Index
AIX@75	Augmentation Index Adjusted to a heart rate of 75 Beats Per Minute
ALTITUDE	Aliskiren Trial in Type 2 Diabetes Using Cardiovascular and Renal Disease Endpoints
AP	Augmentation Pressure
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
ApoL1	Apolipoprotein L1
ARB	Angiotensin Receptor Blocker
ARIC	Atherosclerosis Risk In Communities Study
AUC	Area Under The Curve
AVOID	Aliskiren in The Evaluation of Proteinuria in Diabetes
BMI	Body Mass Index
BP	Blood Pressure
CF-PTT	Carotid Femoral Pulse Transit Time
CF-PWV	Carotid Femoral Pulse Wave Velocity
CGA	Cause, GFR Category and Albuminuria Category
CI	Confidence Interval
CIN	Chronic Interstitial Nephritis
CKD	Chronic Kidney Disease
CRP	C-Reactive Protein
CV	Cardiovascular
CVD	Cardiovascular Disease
CXCL16	Chemokine (C-X-C Motif) Ligand 16
DCCT	Diabetes Control And Complications Trial
DDAH	Dimethylarginine Dimethylaminohydrolase
DKD	Diabetic Kidney Disease
DM	Diabetes Mellitus
ED	Ejection Duration Index
EDIC	Epidemiology Of Diabetes Interventions And Complications
eGFR	Estimated Glomerular Filteration Rate
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicines Agency
EPCs	Endothelial Progenitor Cells
ESAs	Erythropoiesis Stimulating Agents

ESRD	End Stage Renal Disease
EUCLID	EURODIAB Controlled Trial of Lisinopril in Insulin-Dependent Diabetes
FDA	Food and Drug Administration
FEMg	Fractional Excretion of Magnesium
FinnDiane	Finnish Diabetic Nephropathy Study
FMD	Flow-Mediated Dilation
Hb	Hemoglobin
HbsAg	Hepatitis B Virus surface Antigen
HCV	Hepatitis C Virus
HDL	High-Density Lipoprotein
HIV	Human Immunodeficiency Virus
HNF1*Ŭ*	Hepatocyte Nuclear Factor 1***Ŭ***
HOPE	Heart Outcomes Prevention Evaluation study
hsCRP	High Sensitivity C-Reactive Protein
IDL	Intermediate-Density Lipoprotein
IDNT	Irbesartan Diabetic Nephropathy Trial
IDOL	Inducible Degrader Of The LDL Receptor
IFNɔ	Interferon Gamma
IL-6	Interleukin-6
IQR	Inter-Quartile Range
IRMA	Immunoradiometric Assay
KDIGO	Kidney Disease Improving Global Outcomes
KDOQI	Kidney Disease Outcome Quality Initiative
KO	Knockout
LCAT	Lecithin Cholesterol Acyltransferase
LDH	Lactate Dehydrogenase
LDL	Low-Density Lipoprotein
LDLR	LDL Receptor
Leu7Pro	Leucine 7 to Proline 7
LPL	Lipoprotein Lipase
LV	Left Ventricular
LVH	Left Ventricular Hypertrophy
LXR	Liver-X Receptor
MBD	Mineral Bone Disease
MDRD	Modification of Diet In Renal Disease
mTORC1	Mammalian Target Of Rapamycin Complex 1
NADPH	Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
NAE	Nephroangiosclerosis
NF- ə*B*	Nuclear Factor-Kappa B
NHANES	National Health And Nutrition Examination Survey
NID-2	Nephropathy In Diabetes-Type 2
NIDDM	Non Insulin Dependent Diabetes Mellitus
NK	Natural Killer Cell
NKT	Natural Killer T Cell
NMD	Nitroglycerine-Mediated Dilation
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NSAIDS	Non-Steroidal Anti-Inflammatory Drugs

ONTARGET	Ongoing Telmisartan Alone and in combination with Ramipril Global Endpoint Trial
oxLDL	oxidized Low-Density Lipoprotein
P1	Forward Wave
P2	Reflected Wave
PAOD	Peripheral Arterial Occlusive Disease
PCSK9	Proprotein Convertase Subtilisin/Kexin Type 9
PP	Pulse Pressure
PPAR-Ŭ	Peroxisome Proliferator-Activated Receptor Alpha
PPAR-ɔ	Peroxisome Proliferator Activated Receptor Gamma
PRAC	Pharmacovigilance Risk Assessment Committee
PREDIAN	Pentoxifylline for Renoprotection in Diabetic Nephropathy
PTH	Parathyroid Hormone
PWA	Pulse Wave Analysis
PWV	Pulse Wave Velocity
RAAS	Renin Angiotensin Aldosterone System
RAGEs	Receptor for Advanced Glycation End-products
RAS	Renal Artery Stenosis
RENAAL	Reduction of End points in NIDDM with the Angiotensin II Antagonist Losartan study
rHuEPO	recombinant Human Erythropoietin
SD	Standard Deviation
SEVR	Subendocardial Viability Ratio
sICAM-1	soluble Intercellular Adhesion Molecule-1
SLE	Systemic Lupus Erythematous
SNPs	Single Nucleotide Polymorphisms
SRB1	Scavenger Receptor Class B1
SRE	Sterol Responsive Element
SREBP	Sterol Regulatory Element Binding Protein
TGFɓ1	Transforming Growth Factor Beta 1
TIBC	Total Iron Binding Capacity
TNFα	Tumor Necrosis Factor Alpha
UACR	Urinary Albumin Creatinine Ratio
UAER	Urinary Albumin Excretion Rate
UPCR	Urine Protein Creatinine Ratio
VA NEPHRON-D	Veterans Affairs Nephropathy in Diabetes Study
VALIANT	Valsartan in Acute Myocardial Infarction
VDR	Vitamin D Receptor
VLDL	Very Low-Density Lipoprotein
VOP	velocidad de la onda del pulso
VS	Versus
WT	Wild Type
ɑt	Transit Time

SUMMARY

Introduction

Cardiovascular disease (CVD) is common in Chronic Kidney Diseased (CKD) patients and considered to be one of the most common causes of death. However the pathogenic mechanisms linking CKD with CVD remain poorly understood and thus, therapy is unsatisfactory. Both inflammation, abnormal lipid metabolism and bone mineral metabolism abnormalities are thought to play a role. Among different inflammatory factors, chemokine (C-X-C) ligand 16 (CXCL16) is a small cytokine belonging to the CXC chemokine family that has been linked to lipid metabolism and to vascular disease. Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a circulating protein that regulates serum cholesterol levels. Subclinical vascular disease may be studied by a variety of techniques, including ultrasonographic assessment of pulse wave velocity (PWV) and pulse wave analysis (PWA). Arterial PWV is utilized to detect the arterial stiffness.

Hypothesis

The study of biomarkers and the pulse wave assessment will provide insights into the pathogenesis and monitoring of CVD in diabetic CKD patients that may eventually lead to improve outcomes.

Objectives

The general objective of this thesis is to characterize potential markers of vascular injury in patients with CKD which help understand the pathogenesis of vascular injury in CKD in order to develop novel therapeutic approaches, as well as to develop novel monitoring instruments.

1. To study the distribution of plasma values and the correlates of novel plasma biomarkers in diabetic non-dialysis CKD patients.
2. To study the prevalence and correlates of vascular injury as assessed by pulse wave assessment in hypertensive and CKD patients.

Patients and Methods

Patients attending the CKD, diabetic kidney disease (DKD) and hypertension clinics not on dialysis were studied. This cross-sectional observational study assessed baseline data from the following two prospective cohorts of patients.

- A ñbiomarkerò cohort included 134 diabetic patients with CKD. In these patients the novel plasma biomarkers CXCL16 and PCSK9 were assessed.
- A ñPulse Wave Assessmentò cohort included 191 individuals. Of these, 153 were CKD patients and 38 non-CKD patients with hypertension or high cardiovascular (CV) risk. In these patients pulse wave assessment was performed.

Both cohorts had in common 54 CKD patients, for whom both pulse wave assessment and biomarkers data were available.

Results

1. Biomarker cohort

PCSK9

The mean±standard deviation (SD) of plasma PCSK9 levels were 309.8±113.9 ng/ml**.** In multivariate models, the only parameters that remained independently significantly positively correlated with plasma PCSK9 were the therapy with fibrate alone or fibrate with statin, serum total iron binding capacity (TIBC), Log renin and phosphaturia. In general, the multivariate models explain very little of the PCSK9 variability. The best R squared obtained was 0.20.

CXCL16

The mean±SD of plasma CXCL16 levels were 4.0±0.9 ng/ml. Several multivariate models showed that estimated glomerular filtration rate (eGFR) and serum albumin remained independently significantly negatively correlated with plasma CXCL16, while urinary albumin creatinine ratio (UACR) and serum alkaline phosphatase were independently positively correlated with plasma CXCL16. The best R squared obtained was 0.34.

2. Pulse Wave Assessment cohort

Mean PWV

The mean±SD of the PWV were 10.9±3.1 m/sec. Different multivariate models showed that advanced age, systolic BP, DM, serum uric acid, UACR and resin calcium therapy were independently positively correlated with the mean PWV. The best R squared obtained was 0.33.

Delta above upper limit of normal PWV (Delta PWV)

The mean±SD of the Delta PWV were 0.8±1.6 m/sec. In the multivariate analysis, systolic BP, active smoking and resin calcium therapy remained independently positively correlated with delta PWV, while age, urinary potassium and beta blocker therapy were independently negatively correlated with delta PWV. The multivariate model explain a little of the delta PWV variability with R squared of 0.27**.**

Subendocardial viability ratio (SEVR; or Buckberg Index)

In the cohort of diabetic or hypertensive patients, the mean±SD of the SEVR were 141±30 %. In the multivariate analysis, age, beta blocker therapy and log 25 hydroxyvitamin D {25(OH)D} independently positively correlated with SEVR, while female gender, pulse pressure, mean PWV and proton pump inhibitor therapy were independently negatively correlated with SEVR. The multivariate model with the highest R squared was 0.39.

Conclusions

The present thesis allows drawing the following conclusions:

1. Plasma PCSK9 values in diabetic CKD patients are very variable and the factors underlying this variability are unclear. The use of lipid lowering therapies containing fibrates is associated with higher plasma PCSK9. However, PCSK9 did not correlate with features of vascular injury.

2. Plasma CXCL16 values in diabetic CKD patients increased with renal function deterioration and albuminuria and correlate with parameters of bone mineral metabolism, but not with features of vascular injury.

3. Arterial stiffness was very prevalent in CKD patients. Among modifiable factors associated with arterial stiffness we found serum uric acid, albuminuria and potassium metabolism.

RESUMEN

Introducción

La enfermedad cardiovascular (ECV) es común en la enfermedad renal crónica (ERC) y se considera una de las causas más comunes de muerte. Sin embargo, los mecanismos patogénicos que vinculan la ERC con la ECV son poco conocidos y, por tanto, el tratamiento no es satisfactorio. Se cree que tanto la inflamación como alteraciones del metabolismo metabolismo lipídico y óseo-mineral desempeñan un papel importante. Entre los diferentes factores inflamatorios, la quimiocina CXC ligando 16 (CXCL16) se ha relacionado con el metabolismo lipídico y con la enfermedad vascular. La proproteína convertasa subtilisina/kexina tipo 9 (PCSK9) es una proteína circulante que regula los niveles de colesterol en suero. La enfermedad vascular subclínica se puede estudiar por varias técnicas, incluyendo la evaluación ultrasonográfica de la velocidad de la onda del pulso (VOP) y el análisis de la onda del pulso. La VOP se utiliza para detectar la rigidez arterial.

Hipótesis

El estudio de biomarcadores y la evaluación de la onda de pulso proporcionarán información sobre la patogenia y la monitorización de las enfermedades cardiovasculares en los pacientes con ERC diabéticos que eventualmente puede conducir a mejorar su pronóstico.

Objetivos

El objetivo general de esta tesis es caracterizar posibles marcadores de lesión vascular en pacientes con ERC que ayuden a entender la patogenia de la lesión vascular en la ERC con el fin de desarrollar nuevos enfoques terapéuticos, así como desarrollar nuevos instrumentos de monitorización.

1. Estudiar la distribución de los valores plasmáticos y los correlatos de biomarcadores plasmáticos novedosas en pacientes con ERC no dializados diabéticos.
2. Estudiar la prevalencia y los correlatos de la lesión vascular según la evaluación de la onda del pulso en pacientes hipertensos y con ERC.

Pacientes y Métodos

Se estudiaron pacientes de las consultas de ERC, enfermedad renal diabética e hipertensión. En este estudio transversal observacional se evaluaron datos basales de dos cohortes prospectivas de pacientes.

Â Una cohorte "biomarcadores" incluyó 134 pacientes diabéticos con ERC. En estos pacientes se evaluaron los biomarcadores plasmáticos CXCL16 y PCSK9.

Â Una cohorte "evaluación de la onda de pulso" incluyó 191 individuos. De ellos, 153 eran pacientes con ERC y 38 pacientes sin ERC con hipertensión o de alto riesgo cardiovascular. En estos pacientes se realizó la evaluación de la onda de pulso.

Ambas cohortes tenían en común 54 pacientes, de los que disponemos tanto de la evaluación de la onda de pulso como de datos de biomarcadores.

Resultados

1 . Cohorte de biomarcadores

PCSK9

La media±desviación estándar (SD) de los niveles de PCSK9 fue 310±114 ng/ml. En los modelos multivariados, los únicos parámetros que se correlacionaron de forma positiva e independiente con PCSK9 fueron la terapia con fibratos solos o fibratos con estatinas, la trasferrina (TIBC), renina y fosfaturia. En general, los modelos multivariados explican muy poco de la variabilidad de PCSK9. La mejor R cuadrado obtenida fue de 0,20.

CXCL16

La media±SD de los niveles de plasma CXCL16 fue 4,0±0,9 ng/ml. Varios modelos multivariados mostraron que la tasa de filtración glomerular y la albúmina sérica se correlacionan negativamente de forma independiente y significativa con CXCL16, mientras que el índice albúmina creatinina urinaria (UACR) y la fosfatasa alcalina en suero se correlacionaron positivamente con CXCL16. La mejor R cuadrado obtenida fue de 0,34.

2 . Cohorte de evaluación de onda del pulso

Velocidad de la onda del pulso

La media±SD de la VOP fue 10,9±3,1 m/sec. Diferentes modelos multivariados mostraron que la edad avanzada, la presión arterial sistólica, la diabetes mellitus, el ácido úrico en suero, UACR y el tratamiento con resincalcio se correlacionaron positivamente de forma independiente con la VOP. La mejor R cuadrado obtenida fue de 0,33.

Delta por encima del límite superior de la normalidad de VOP (Delta VOP)

La media±SD de la Delta VOP fue 0,8±1,6 m/sec. En el análisis multivariado, la presión arterial sistólica, el tabaquismo y el resincalcio se correlacionaron de forma independiente y positiva con la Delta VOP, mientras que la edad, potasio urinario y beta bloqueantes se correlacionaron de forma independiente y negativa con la Delta VOP. El modelo multivariado explica un poco de la variabilidad de la Delta VOP con R cuadrado de 0,27.

Coeficiente de viabilidad subendocárdica (SEVR) [Índice Buckberg]

En la cohorte de pacientes diabéticos o hipertensos, la media ± SD de la SEVR fue 141±30%. En el análisis multivariado, la edad, la terapia con beta bloqueantes y la 25 hidroxivitamina D {25(OH)D} se correlacionaron de forma independiente y positiva con SEVR , mientras que el sexo femenino, la presión del pulso, la VOP y la terapia con inhibidores de la bomba de protones se correlacionaron de forma independiente y negativa con SEVR. El modelo multivariado con el mayor R cuadrado fue 0,39.

Conclusiones

La presente tesis permite extraer las siguientes conclusiones:

1. Los valores de PCSK9 plasmáticos en pacientes con ERC diabéticos son muy variables y los factores que subyacen a esta variabilidad no están claros. El uso de terapia hipolipemiante con fibratos se asocia con niveles de PCSK9 más altos. Sin embargo, PCSK9 no se relacionó con patología cardiovascular.

2. Los valores de CXCL16 plasmáticos en pacientes diabéticos con ERC aumentan con el deterioro de la función renal y la albuminuria, y se correlacionaron con parámetros del metabolismo mineral óseo, pero no con patología cardiovascular.

3. La rigidez arterial es muy frecuente en los pacientes con ERC. Entre los factores modificables asociados con la rigidez arterial se encuentran la uricemia, albuminuria y el metabolismo de potasio.

Introduction

1. Chronic Kidney Disease (CKD)

CKD is a worldwide public health problem with an increasing incidence and prevalence, poor outcomes, and high cost. In the United States, at the end of 2011, 615,899 dialysis and transplant patients were receiving treatment for end-stage renal disease (ESRD); a 3.2% increase from 2010 (Figure 1) [1]. Outcomes of CKD include not only kidney failure but also complications of decreased kidney function and CVD. Current evidence suggests that some of these adverse outcomes can be prevented or delayed by early detection and treatment [2]. Unfortunately, CKD is underdiagnosed and undertreated, in part, until recently, as a result of lack of agreement on a definition and classification of its stages of progression.

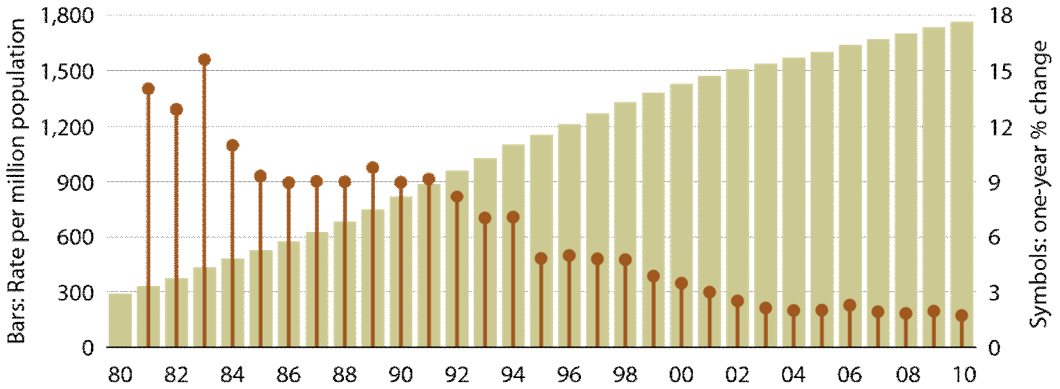


Figure 1: Adjusted prevalent rates of ESRD and annual percent change in the United States

Kidney Disease Outcome Quality Initiative (KDOQI) guidelines 1) define CKD and classify its stages, regardless of underlying cause, 2) evaluate laboratory measurements for the clinical assessment of kidney disease, 3) associate the level of kidney function with complications of CKD, and 4) stratify the risk for loss of kidney function and development of CVD.

CKD is defined as kidney damage or eGFR below 60 ml/min/1.73 m^2 or other evidence of kidney injury for 3 months or more irrespective of the cause. KDOQI guidelines have classified CKD into 5 stages (Table 1) [3].

Table 1: CKD stages

Stage	Description	GFR (ml/min/1.73m^2)
1	Kidney damage with normal or ↑GFR	≥ 90
2	Kidney damage with mild ↓GFR	60 - 89
3	Moderate ↓GFR	30 - 59
4	Severe ↓GFR	15 – 29
5	Kidney failure	< 15 (or dialysis)

More recent Kidney Disease Improving Global Outcomes (KDIGO) guideline defined CKD as abnormalities of kidney structure or function, present for more than 3 months, with implications for health and CKD is classified based on cause, GFR category, and albuminuria category (CGA) (Table 2) [4].

Table 2: Recent KDIGO CKD staging and prognosis

GFR categories (ml/min/1.73m^2) Description and range				Persistent albuminuria categories Description and range		
				A1	**A2**	**A3**
				Normal to mildly increased	Moderately increased	Severely increased
				< 30 mg/g < 3 mg/mmol	30-300 mg/g 3-30 mg/mmol	> 300 mg/g > 30 mg/mmol
	G1	Normal or high	≥ 90			
	G2	Mildly decreased	60-89			
	G3a	Mildly to moderately decreased	45-59			
	G3b	Moderately to severely decreased	30-44			
	G4	Severely decreased	15-29			
	G5	Kidney failure	<15			

Green: low risk (if no other markers of kidney disease, no CKD); Yellow: moderately increased risk; Orange: high risk; Red: very high risk

Factors affecting initiation and progression of CKD

CKD is likely to be a multi-hit process. Risk factors for CKD include susceptibility, initiation, and progression factors. Susceptibility factors predispose to CKD, whereas initiation factors directly trigger kidney damage. Progression factors are associated with worsening of already established kidney damage. The aim of identifying susceptibility and initiation factors for CKD is to define individuals at high risk for development of CKD; with progression factors, the aim is to define individuals at high risk for worsening CKD and subsequent loss of kidney function (Table 3) [5]. Once it is established, CKD progression is influenced by a number of modifiable and nonmodifiable risk factors [6].

Table 3: Summary of risk factors associated with the initiation and progression of CKD

Initiation Factors	Progression Factors
• Systemic Hypertension • Diabetes Mellitus • Cardiovascular Disease • Dyslipidemia • Obesity / Metabolic Syndrome • Hyperuricemia • Smoking • Low socioeconomic status • Nephrotoxic exposure: non-steroidal anti-inflammatory drugs (NSAIDS), analgesics, traditional herbal use, heavy metal exposure (such as lead)	• Older age • Gender (male) • Race / Ethnicity • Genetic predisposition • Poor blood pressure control • Poor glycemia control • Proteinuria • Cardiovascular disease • Dyslipidemia • Smoking • Obesity / Metabolic Syndrome • Hyperuricemia • Low socioeconomic status • Alcohol consumption • Nephrotoxins; NSAIDS, Radiocontrast material • Acute Kidney Injury

Nonmodifiable progression risk factors

Age: The prevalence of CKD rises dramatically with age. Based on the results of the National Health and Nutrition Examination Survey 1999-2004 (NHANES), more than one third of those aged 70 or older have moderate or severe CKD defined as an eGFR <60 ml/min/1.73 m^2 [7]. While all stages of CKD are more common at older ages, it is the prevalence of moderate CKD (eGFR 30-59 ml/min/1.73 m^2) that increases most dramatically with advancing age.

Gender: The progression rate of many renal diseases is affected by sex [8]. A meta-analysis of more than 11000 patients from 68 different studies, demonstrated that renal disease in women with polycystic kidney disease, IgA nephropathy, membranous glomerulopathy, and óCKD of unknown aetiologyô progresses at a slower rate than it does in blood pressure (BP)- and lipid levels-matched men with these diseases [9]. Two additional population-based studies showed that men were associated with a worse CKD progression than women [10, 11].

Race: In the United States, for all causes of ESRD, African Americans have a faster rate of progression than Caucasians. The incidence and prevalence of diabetic and hypertensive CKD are higher in African and Hispanic Americans compared with Caucasians [12].

Genetics: Studies of linkage and association analyses with candidate gene approaches have demonstrated that genetic factors play a crucial role in CKD and ESRD. Recent technologic advancements in genome-wide association studies are likely to uncover new CKD susceptibility genes [13].

Modifiable progression risk factors

Hypertension: Systemic hypertension is an important cause, consequence, and presenting feature of CKD. It is thought to be one of the leading causes of ESRD worldwide [14]. Experimental and epidemiologic studies have shown that sustained hypertension is indeed a significant contributor to the progression of CKD [15]. It is believed that the transmission of systemic hypertension into the glomerular capillary beds and the resulting glomerular hypertension contribute to the progression of glomerulosclerosis.

Lipids: Dyslipidemia may contribute to glomerulosclerosis and tubulointerstitial fibrosis. A number of studies of diabetic and non-diabetic nephropathies have confirmed by multivariate analysis that dyslipidemia is a risk factor for a faster rate of CKD progression [15].

Proteinuria: A large number of studies in patients with diabetic and non-diabetic glomerular and nonglomerular diseases confirmed, by multivariate analysis, that heavy proteinuria is associated with a faster rate of CKD progression [16, 17]. Furthermore, reduction of proteinuria by diet, angiotensin-converting enzyme inhibitor (ACEI), or angiotensin receptor blocker (ARB) predicts a better outcome [17]. The extent of reduction in proteinuria is often proportional to the benefit accrued by such intervention on CKD progression.

Albuminuria, CKD, and CVD: Urinary albumin excretion rate (UAER) is independently associated with the presence and severity of CVD in the general population [18]. Even low-grade albuminuria (below the current microalbuminuria threshold UACR <30 mg/g) in middle-aged non-diabetic and non-hypertensive individuals is associated with increased CV risk. Diffuse endothelial and vascular dysfunction may be the common pathway linking albuminuria to the manifestations and prognosis of CKD and CVD. Albuminuria has been linked in a number of studies to underlying systemic atherosclerosis, diffuse vascular stiffness, and maladaptive vascular remodeling [19, 20].

CKD is now defined as a CV risk equivalent, and patients with moderate to severe CKD are taken to be in the ñhighest risk groupò for development of CVD [16, 17]. Patients with CVD are also at a higher risk for development of CKD. Overall, these observations may, in part, be explained by the fact that CVD and CKD share many risk factors, including obesity, metabolic syndrome, hypertension, diabetes mellitus (DM), dyslipidemia, and smoking. In addition, CVD may have direct hemodynamic effects on the kidneys that may promote initiation and progression of CKD, including decreased kidney perfusion in heart failure and atherosclerosis of the renal arteries, with subsequent ischemic nephropathy. Evidence is linking faster rate of CKD progression to severe atherosclerotic disease [21].

Renin-Angiotensin-Aldosterone System (RAAS): The links between systemic hypertension, proteinuria/albuminuria, and CVD may be mediated in part by changes in the RAAS in CKD. A number

of experimental and clinical data have implicated the RAAS in the pathogenesis of hypertension, proteinuria, and renal fibrosis throughout the course of CKD. Consequently, interventions aimed at inhibition of the RAAS have been proved to be extremely effective in slowing the progression of CKD [22].

Glycemia: Randomized clinical trials have demonstrated that tight diabetes control can potentially slow the rate of progression of diabetic microvascular complications, including diabetic nephropathy in both type 1 and type 2 DM [23].

Inflammation and CKD: Cross-sectional studies show that patients with CKD have a pronounced inflammatory phenotype, including an elevated serum concentration of inflammatory markers, such as C-reactive protein (CRP) and interleukin (IL)-6, and decreased serum albumin levels [24, 25]. A retrospective analysis of data from 9,250 adults enrolled in NHANES II confirmed systemic inflammation as an independent risk factor for future development of CKD [26].

Obesity: Several studies have linked obesity, and the associated metabolic syndrome, with increased risk of CKD. Anecdotal reports suggest that weight reduction reduces obesity-related renal hemodynamic changes as well as CKD-associated proteinuria, and increased weight gain is associated with its progression [27].

Smoking: Smoking has been shown to increase the risk of albuminuria as well as that of progression of CKD. Possible mechanisms whereby cigarette smoking may contribute to kidney damage include sympathetic nervous system activation, hypertension, endothelial injury, and potential direct tubulotoxicity [28].

Uric Acid: Hyperuricemia has been associated with systemic hypertension, CVD, and CKD [29]. Hyperuricemia may cause hypertension and renal injury through crystal-independent pathways, notably a stimulation of the RAAS. However, not all observational studies confirm the association between uric acid and CKD progression. An observation from the United States suggested that in patients with CKD, hyperuricemia appears to be an independent risk factor for all-cause and CVD mortality but not for kidney failure [30]. Nevertheless treating hyperuricemia slowed progression of CKD in an open label randomized controlled trial from Spain [31].

2. Diabetic kidney disease (DKD)

Around 10% of the population of Western countries is diabetic. The current obesity epidemic is expected to increase the incidence of type 2 DM, which accounts for 90% of DM cases. In the United States, 40% of adults with DM have some degree of CKD [32]. Prevalence of DKD increased from 1988 to 2008 in proportion to the prevalence of DM, from 2.2% in NHANES III to 3.3% in NHANES 2005-2008. Among persons with DM, prevalence of DKD was stable despite increased use of glucose-lowering medications and RAAS inhibitors [33]. DKD is characterized by proteinuria and progressive loss of renal function leading to ESRD requiring renal replacement therapy. The pathological substrate of DKD is a progressive loss of parenchymal renal cells, inflammation and extracellular matrix accumulation. DKD remains the most frequent cause of ESRD in developed countries. This means that current nephroprotective strategies, based on targeting the RAAS are insufficient to stop progression of the disease in a sizable number of DM patients. Only a comprehensive understanding of the molecular basis for renal injury initiation and progression will allow the rational design of novel, more successful therapeutic strategies [34].

The expanding spectrum of DKD: non-albuminuric renal insufficiency

Microalbuminuria (UAER between 30 and 300 mg/day or mg/g creatinine) is considered the earliest clinical marker of DKD. Microalbuminuria precedes the development of macroalbuminuria (UAER > 300 mg/day or mg/g creatinine). The onset of macroalbuminuria is usually followed by a slowly progressive decline in GFR and ESRD [35]. Albuminuria occurs secondary to podocyte injury (podocytopathy) [36]. DM causes hypertrophy of podocytes, and initial relative and subsequent absolute podocytopenia due to initial increase in glomerular volume and later detachment or apoptosis of podocytes [37]. Normoalbuminuric patients are not completely protected from DKD, which may develop insidiously and progress silently to renal failure [38]. Around 25 to 35% of diabetic patients with impaired GFR have no increased albuminuria [39, 40]. In NHANES III, among adults with type 2 DM and CKD, only 45% and 19% had microalbuminuria and macroalbuminuria, respectively. The population estimate of adults with

type 2 DM and CKD in the absence of diabetic retinopathy or albuminuria was approximately 0.3 million. In type 1 DM patients enrolled in the Epidemiology of Diabetes Interventions and Complications (EDIC)/Diabetes Control and Complications Trial (DCCT) studies the cumulative incidence of stage 3 CKD (eGFR <60 ml/min/1.73 m^2) was 11.4% over 14-19 years. Of patients developing stage 3 CKD 24% had albuminuria <30 mg/24h at all prior evaluations, 16% had developed microalbuminuria (30-300 mg/24 h) and 61% had macroalbuminuria (>300 mg/24 h) before they reached stage 3 CKD [39]. In the EDIC study ten-year cumulative incidences of progression to macroalbuminuria, impaired GFR, ESRD, and regression to normoalbuminuria were 28%, 15%, 4%, and 40%, respectively. After the development of persistent microalbuminuria, intensive glycemic control, lower BP, and a more favorable lipid profile are associated with improved outcomes [35]. The rate of loss of GFR is lower in patients with stable microalbuminuria or regression to normal albuminuria compared to patients who progressed to macroalbuminuria [39]. The factors responsible for GFR loss in non-proteinuric DKD patients have not been identified [41, 42]. Intrarenal vascular disease may contribute. However, non-proteinuric DKD also occurs in type 1 DM patients who are generally younger and less likely to have high intrarenal vascular resistance [43]. Genetic backgrounds may prevent development of proteinuria in face of DKD. Low-grade chronic inflammation may also contribute to DKD [44-46]. Elevated serum inflammatory markers are associated with decline of GFR in nonproteinuric DKD patient, independent of UAER [47].

The conundrum of DKD management

Current treatment strategies for DKD include lifestyle improvements (cessation of smoking and dietary modifications), glycemic control, cholesterol management and reduction of BP [32]. The 2012 update of American Diabetes Association (ADA) guidelines [48] recommends optimization of glycemic and BP control to reduce the risk of progression of DKD as the cornerstone of the treatment (A evidence). Lowering HbA1C to below or around 7% has been shown to reduce microvascular and neuropathic complications of type 1 and type 2 DM. Postprandial glucose may be targeted if HbA1C goals are not met despite reaching preprandial glucose goals. Glycemic management of type 2 DM has been recently reviewed [49] and focused on drug therapy aimed at preventing, slowing or regressing DKD beyond glycemic control. Hypertension is common in diabetic patients, even in the absence of DKD. The recommended BP target in diabetic patients is 125/75 mmHg to prevent both DKD progression and CV events [48]. ACEIs or ARBs are the elective drugs to treat hypertension in DKD (evidence A). Other drugs to control BP (diuretics, calcium channel blockers, beta-blockers or alpha-blockers) may be used for adequate BP control in diabetic patients under RAAS inhibitors [50].

RAAS targeting: ACEI, ARB, renin inhibitors and mineralocorticoid receptors blockers are designed to block the RAAS system. ACEIs and ARBs have been most studied in the setting of hypertension in diabetes. In diabetic patients with micro- or macroalbuminuria, either ACEIs or ARBs decreased urinary protein excretion in a dose-dependent manner and should be given at the maximum dose which could be tolerated [51, 52]. There are no adequate comparisons of ACEIs vs. ARBs. However in clinical trials, ACEIs delay the progression of nephropathy in patients with type 1 DM, hypertension, and any degree of albuminuria [53], both ACEIs and ARBs delay the progression to macroalbuminuria in patients with type 2 DM, hypertension, and microalbuminuria, and ARBs delay the progression of nephropathy in patients with type 2 DM, hypertension, macroalbuminuria, and renal insufficiency (eGFR<60 ml/min/1.73m^2) [48, 54, 55].

A further concept is DKD prevention in normotensive, normoalbuminuric patients [56]. The role of RAAS blockade to prevent the appearance of microalbuminuria in normotensive, normoalbuminuric patients is not well defined. Perindopril delayed the increase in albuminuria in 89 normotensive normoalbuminuric type 1 DM patients [57]. In 156 normotensive, normoalbuminuric patients with type 2 DM, ACEI reduced transition to microalbuminuria from 19% placebo to 6.5% enalapril in 6 years of follow-up [58]. EUCLID (EURODIAB Controlled Trial of Lisinopril in Insulin-Dependent Diabetes) reported a non-significant reduction in incidence of increased albuminuria in 530 normotensive patients with type 1 DM [59]. The DIRECT-Renal investigators studied 5231 diabetics and concluded that candesartan had no effect on the primary prevention of increased urinary albumin excretion during 4.7 years in normoalbuminuric, normotensive patients with type 1 DM or normoalbuminuric patients with type 2 DM who were either normotensive or hypertensive [60]. The RASS investigators studied 288 normotensive, normoalbuminuric type I diabetics with renal biopsies and concluded that there were no structural or functional benefits to the kidney from blockade of the RAAS with either an ACEI or ARB [61].

While targeting the RAAS, it is important to monitor serum creatinine and potassium since there is a risk of hyperkalemia or impairment of renal function. Targeting the system sets off compensatory mechanisms that may increase angiotensin II, aldosterone or renin and may account for delayed increases

in albuminuria following an initial decrement. Thus, hyperplasia of the renin-secreting juxtaglomerular apparatus has been described in experimental animals and in humans [62]. It has been hypothesized that partial RAAS blockade lies behind the failure to prevent progression in some DKD patients. In this regard, dual blockade of the RAAS has been proposed to overcome compensatory mechanisms. However, dual or triple blockade of the RAAS increases the risk of adverse events such as hyperkalemia or hypotension, especially in older patients [63].

Combined RAAS blockade: Combination of different drugs targeting the RAAS decreases albuminuria more than just one drug [64, 65]. However safety concerns have been raised by a number of studies, including ONTARGET (Ongoing Telmisartan Alone and in combination with Ramipril Global Endpoint Trial) and ALTITUDE (Aliskiren Trial in Type 2 Diabetes Using CV and Renal Disease Endpoints) and the efficacy to impact long-term renal function has not yet been demonstrated. COOPERATE is a key trial exploring the ACEI/ARB combination in non-diabetic kidney disease that showed protection from progression of CKD in 3 years. However it was withdrawn after serious concerns about the authenticity of the results were raised [66]. A 2006 meta-analysis on the combination of ACEIs and ARBs in renal disease disclosed reduction of proteinuria and renoprotection in patients with proteinuric CKD [67]. However the benefits of double blockade of RAAS were greater in patients with non-diabetic renal disease than in DKD despite similar BP control.

The ONTARGET trial studied patients older than 55 years that had diabetes with organ damage and vascular disease. Subjects were treated with telmisartan, ramipril or the combination for a median of 56 months. Double blockade resulted in lower BP and proteinuria, but the incidence of hyperkalemia and the incidence of renal replacement (dialysis, transplantation) or death were increased [63]. The VA NEPHRON-D (Veterans Affairs Nephropathy in Diabetes study) trial did not evidence advantages of lisinopril/losartan over losartan alone regarding CKD progression in terms of reduction of eGFR, in a total of 1850 patients with overt proteinuria [68].

The direct renin inhibitor aliskiren is Food and Drug Administration (FDA) approved as an anti-hypertensive medication. Aliskiren on top of optimal antihypertensive treatment including an ARB reduced urinary albumin excretion 20% in microalbuminuric type 2 DM patients in the Aliskiren in the evaluation of proteinuria in diabetes (AVOID) trial [69, 70]. However, a metanalysis raised concerns on the excess occurrence of hyperkalemia in patients enrolled in clinical trials [71]. The ALTITUDE trial was terminated because patients randomized to aliskiren experienced a higher incidence of kidney impairment, hypotension and hyperkalemia. ALTITUDE randomized 8606 high risk type 2 DM patients to receive aliskiren or placebo added to recommended cardio-renal protective treatment including ACEIs or ARBs, but not both [72]. Primary outcome measures included CV events as well as ESRD and doubling of serum creatinine.

Aldosterone has direct effects promoting inflammation and fibrosis. Despite no changes in blood glucose levels or BP, spironolactone significantly decreased urinary albumin excretion when used alone [73]. Spironolactone also has an additive effect on proteinuria when used in combination with an ACEI or an ARB in both type 1 and type 2 DM [74]. Co-administration of the aldosterone antagonist eplerenone with an ACEI in type 2 DM decreased UAER compared to the placebo group. There was a significant increase in hyperkalemia episodes with the higher (100 mg/d), but not the lower (50 mg/d) eplerenone dose [75]. However, the effect on long-term preservation of renal function by aldosterone targeting has not been addressed.

In April 2014, the Pharmacovigilance Risk Assessment Committee (PRAC) of the European Medicines Agency (EMA) has advised that combining different classes of medicines that act on RAAS should not be recommended, and in particular that patients with DKD should not be given an ARB with an ACE-inhibitor. Where such combination (dual blockade) is considered absolutely necessary, it must be carried out under specialist supervision with close monitoring of kidney function, fluid and salt balance and blood pressure. (This would include the licensed use of the ARBs candesartan or valsartan as add-on therapy to ACE-inhibitors in patients with heart failure who require such a combination.) The combination of aliskiren with an ARB or ACE-inhibitor is strictly contraindicated in those with kidney impairment or diabetes (EMA/196502/2014) [76].

Beyond the RAAS: Given the limitations of RAAS blockade, attempts have been made to improve the outcome of DKD using different add-on strategies. Recent clinical trials have targeted albuminuria or GFR by adding to conventional ARB- or ACEI-based therapy drugs such as vitamin D receptor (VDR) activators, bardoxolone, endothelin receptor antagonists, pirfenidone, pyridoxamine, sulodexide, pentoxifylline or ruboxistaurin [77-84].

3. Cardiovascular Risk in Diabetic CKD

Cardiac diseases are independently associated with a deterioration of renal function and worsening of existing kidney disease. On the other hand, CKD is an independent risk factor for increased CV morbidity and mortality [85]. CV and total mortalities are 10- to 100-fold higher in ESRD patients than in age-matched controls. Indeed, the risk of CV death of a 30-year-old ESRD patient is similar to that of an 80-year-old in the general population [86, 87]. Thus, ESRD may be considered an accelerated aging syndrome. CVD in CKD has a complex pathogeneses, and traditional risk factors are not able to fully explain its high incidence and prevalence [88] and interventions that have been successful in the general population have failed to increase survival in ESRD patients [89, 90].

Both albuminuria and GFR are believed to be risk factors for CV events. There are limited data as to whether these two factors are associated with adverse outcomes independently not only of other known CV risk factors but also of each other in patients with type 2 DM [91]. The Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE) study [92] showed that high albuminuria and low GFR are independent risk factors for CV events among patients with type 2 DM. However, the two main clinical features associated with DKD, diabetic retinopathy and albuminuria, were detected in only 7.1 and 29.3% of patients, respectively [93] and 62% of patients with a GFR <60 mL/min did not have concurrent albuminuria [92].

The Nephropathy In Diabetes-Type 2 (NID-2) study [94] was originally designed to investigate the prevalence of CV risk factors, their management and the achievement of international guideline targets in a large population of type 2 DM patients with a clinical diagnosis of classical diabetic nephropathy defined by the concomitance of albuminuria and severe diabetic retinopathy, followed up in the tertiary care setting. The cross-sectional phase of the NID-2 study pointed out that patients with diabetic nephropathy are characterized by clusters of risk factors, not at target, compatible with a high CV risk profile [94].

Cardiovascular disease in diabetic patients with macroalbuminuria

The role of macroalbuminuria in CVD has been outlined in the post-hoc analyses of the Irbesartan Diabetic Nephropathy Trial (IDNT) and the Reduction of End points in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) studies. The IDNT enrolled subjects with type 2 DM, hypertension, and macroalbuminuria [54]. Irbesartan proved to be superior to amlodipine or placebo with respect to renoprotection but no difference was detected between treatment groups on the secondary outcome of CV events. A post hoc analysis performed to assess the relationship between baseline UACR and the CV composite (CV death, nonfatal myocardial infarction, hospitalization for heart failure, stroke, amputation, and coronary and peripheral revascularization) revealed in a multivariate analysis that albuminuria is an independent risk factor for CV events with a 1.3-fold increased relative risk for each natural log increase of 1 U in UACR [95].

Similar results were noted in the RENAAL study whereby losartan was superior to placebo with regards to renoprotection in type 2 diabetic, hypertensive patients with macroalbuminuria. However losartan conferred no statistically significant benefit on the secondary CV outcomes, although de novo heart failure was less frequently noted in the losartan group [96]. Nevertheless, in a post-hoc analysis of RENAAL, baseline albuminuria was again shown to be a predictor of both the prespecified composite CV end point (composite of myocardial infarction, stroke, first hospitalization for heart failure or unstable angina, coronary or peripheral revascularization, or CV death) as well as of heart failure alone. With subjects stratified into 3 groups on the basis of baseline UACR, comparison of the highest tertile with the lowest revealed an adjusted hazard ratio of 1.92 for the composite CV end point and 2.70 for heart failure. In multivariate analysis, baseline albuminuria was the strongest independent predictor of both outcomes. In addition, the change in UACR from baseline to 6 months was the only dynamic correlate of adverse CV outcomes. A 50% reduction in baseline albuminuria translated into an 18% reduction in the composite CV end point and a 27% reduction in the risk of heart failure. It has thus been suggested that albuminuria serve as an indicator of therapeutic response.

Cardiovascular disease in diabetic patients with microalbuminuria

Microalbuminuria also correlates with adverse CV events. In type 2 DM microalbuminuria was the strongest predictor of adverse CV outcomes with an odds ratio of 10.02 [97]. In addition, in the Heart Outcomes Prevention Evaluation (HOPE) study, a baseline UACR >20 mg/g (present in 33% of the diabetic cohort and in 15%of the non-diabetic cohort) increased the adjusted relative risk of CV events (1.83), all-cause death (2.09), and hospitalization for congestive heart failure (3.23). In addition, the impact of microalbuminuria on the primary composite outcome of CV death, myocardial infarction, or

stroke was significant in both diabetic (relative risk 1.97) and non-diabetic (relative risk 1.61) subjects [98].

Cardiovascular disease in diabetic patients with low GFR

Declining renal function plays an important role in CVD in diabetic patients, as demonstrated in the Valsartan in Acute Myocardial Infarction (VALIANT) trial; a multicentre double-blind randomized controlled trial which randomly assigned patients to receive captopril, valsartan, or both. GFR was estimated using the 4-component Modification of Diet in Renal Disease (MDRD) equation. The likelihood of experiencing the primary end-point of all-cause mortality was higher in patients with than without DM for each level of renal function. In patients with DM, a decrease in eGFR of 10 units was associated with a hazards ratio of 1.09 (95% confidence interval (CI) 1.06 to 1.12, $p < 0.001$) for risk of fatal and nonfatal CV outcomes independent of treatment assignment [99]. The importance of renal impairment in predicting outcome following myocardial infarction in diabetic patients was also investigated and shown that the combination of renal impairment and DM was associated with a particularly high risk of myocardial infarction and death/ myocardial infarction following a non-ST elevation myocardial infarction, suggesting that attention to preserving renal function may be of particular benefit for reducing CV risk in diabetic patients [100].

DKD and peripheral arterial occlusive disease

Peripheral arterial occlusive disease (PAOD) is a major cause of morbidity. PAOD carries a particularly poor prognosis in DM [101, 102]. DKD has been reported to be associated with PAOD in both type 1 and type 2 DM subjects [103]. Both albuminuria and declining GFR are probably associated with increased risk. PAOD is more prevalent in type 2 DM patients with increased UACR [104, 105]. It is likely that microalbuminuria reflects widespread vascular damage. In addition, a reduction in GFR is associated with PAOD in both non-diabetic [106] and diabetic patients [104, 105]. Albuminuria and declining renal function are also independent predictors of the occurrence of PAOD in non-Caucasian populations in both genders [107]. Interestingly, in a type 2 DM populations with proliferative diabetic retinopathy declining renal function was an independent predictors of PAOD, whilst microalbuminuria was not [108, 109].

In type 2 DM patients with normal ankle-brachial indices (ABIs), eGFR correlated positively with flow volume and negatively with ankle-brachial PWV and with resistive index in patients with albuminuria but not in those with normoalbuminuria [110]. This suggests an association between nephropathy and impaired lower limb circulation secondary to higher arterial stiffness and increased vascular resistance in type 2 DM. In Japanese type 2 DM patients with normal ABIs the major risk factors for reduced flow volume were age, hypertension, and DKD [111]. These results suggest that DKD may plays a role in the early stages of PAOD in diabetes by altering vascular resistance and arterial stiffness.

Cardiovascular consequences of DKD

Disturbed renal function in DKD leads to a multitude of adverse CV effects (Figure 2) [109]. Increased BP together with accelerated large vessel disease and increased arterial stiffening and calcification lead to increased afterload whilst hypervolemia and anemia lead to increased preload with consequent adverse effects on the heart. These cardiac abnormalities are further augmented by the local activation of the RAAS and endothelin system, amongst others. The RAAS plays a role in the activation of the sympathetic nervous system, the dysregulation of endothelial function and progression of atherosclerosis, and inhibition of the fibrinolytic system, while direct profibrotic actions of angiotensin II and aldosterone in the kidney and heart promote end-organ injury [112].

High blood pressure predisposes CVD, CKD and predates the onset of DKD. However high BP is also a consequence of renal disease, thereby initiating a vicious cycle resulting in progression of CKD as well as in CKD being causally related to increased CV risk [113].

Left ventricular hypertrophy (LVH) is an important risk factor for adverse CV outcomes in patients with CKD and in type 2 DM subjects with and without DKD [114]. In addition, LVH increases with progression of nephropathy [115, 116]. Multiple factors may contribute to LVH, including high BP, vascular stiffening, anemia, CV autonomic neuropathy, uremia toxins and albuminuria [117, 118].

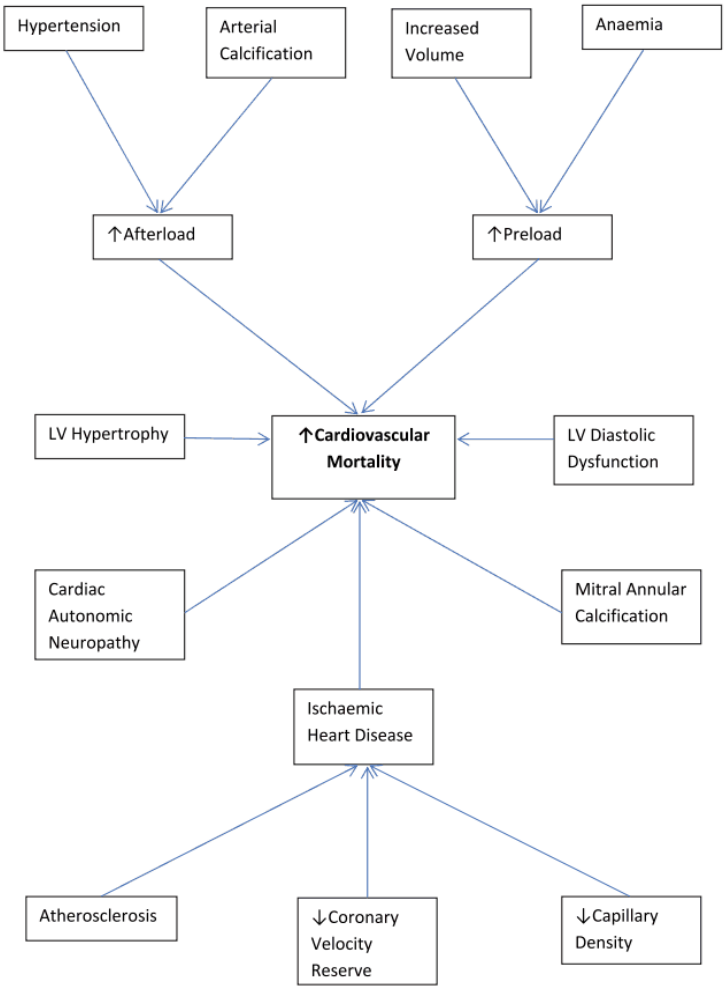


Figure 2: Cardiovascular Consequences of DKD

Left ventricular (LV) diastolic dysfunction is another adverse cardiac effect. DKD is a significant predictor of LV diastolic dysfunction in CKD subjects, independent of other influencing factors such as age, BP, renal function, anemia and LVH. LV diastolic dysfunction is characterized by elevated LV end-diastolic pressure or left arterial pressure, ultimately leading to LV systolic dysfunction and overt heart failure symptoms [119].

Mitral annular calcification predicts atrial fibrillation, stroke and CV disease morbidity and mortality. In the Framingham Heart Study, the combination of both CKD and mitral annular calcification was associated with a three-fold increased risk of death compared with those without CKD or mitral annular calcification ($p<0.01$), following adjustment for age and gender. However, no significant association was found between CKD and either aortic sclerosis or aortic annular calcification [120].

Ischemic heart disease is strongly associated with DKD. Patients with both ESRD and DM carry an increased risk of coronary atherosclerosis. In addition to coronary atherosclerosis, abnormalities in myocardial blood flow and in coronary flow reserve could be responsible for CV morbidity in DKD. In the presence of end-organ damage caused by DM, abnormalities in coronary physiology may be seen in the absence of overt epicardial coronary artery disease [121].

In addition to arterial changes, reduction in capillary density interferes with oxygen delivery in CKD. In uremic subjects with LVH, there is a significant decrease in capillary density, leading to an increase in inter-capillary distance, and potentially compromising the blood and oxygen supply of the myocardium under conditions of increased demand, thus rendering the myocardium more prone to ischemic injury [122].

Potential pathogenic mechanisms

Various mechanisms account for the CV dysfunction present in renal disease, as outlined below.

Endothelial cell dysfunction: The vascular endothelium is a versatile multifunctional tissue having many synthetic and metabolic properties (Figure 3) [109].

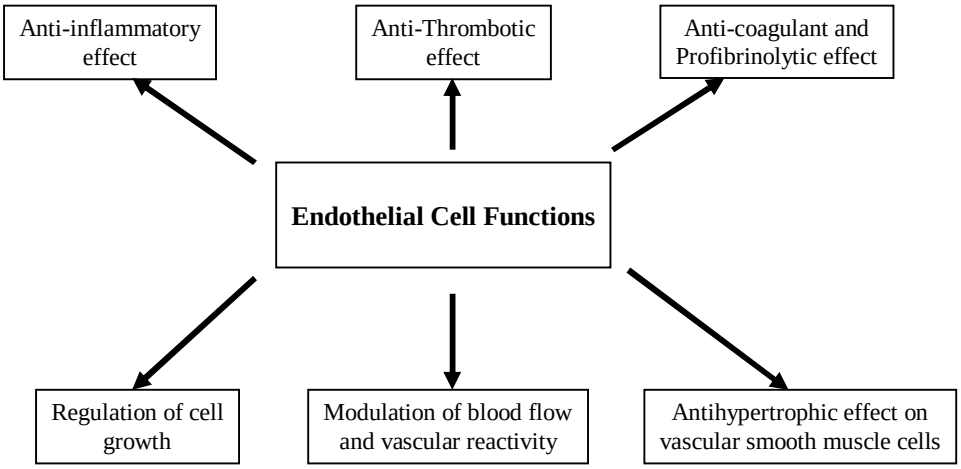


Figure 3: Endothelial Cell Properties and Functions

Endothelial cell dysfunction seems to be central in the genesis of many different aspects of CV dysfunction in subjects with DKD, such as increased PWV [123] and decreased flow-mediated dilation (FMD) of the brachial artery [124]. The various factors leading to endothelial cell dysfunction have been outlined in (Figure 4) [125]. Endothelium-dependent vascular dysfunction, as assessed using FMD, correlates with proteinuria in type 2 DM subjects with early DKD. Interestingly, no correlation was found between proteinuria and nitroglycerine-mediated dilation (NMD) of the brachial artery. This suggests that the bioavailability of nitric oxide (NO) is decreased, but that the vessels remain responsive to NO in DKD. Proteinuria might lead to endothelial dysfunction via increasing serum levels of the nitric oxide synthase (NOS) inhibitor asymmetrical dimethylarginine (ADMA), together with loss of vasoprotective circulating proteins such as adiponectin and fetuin [124].

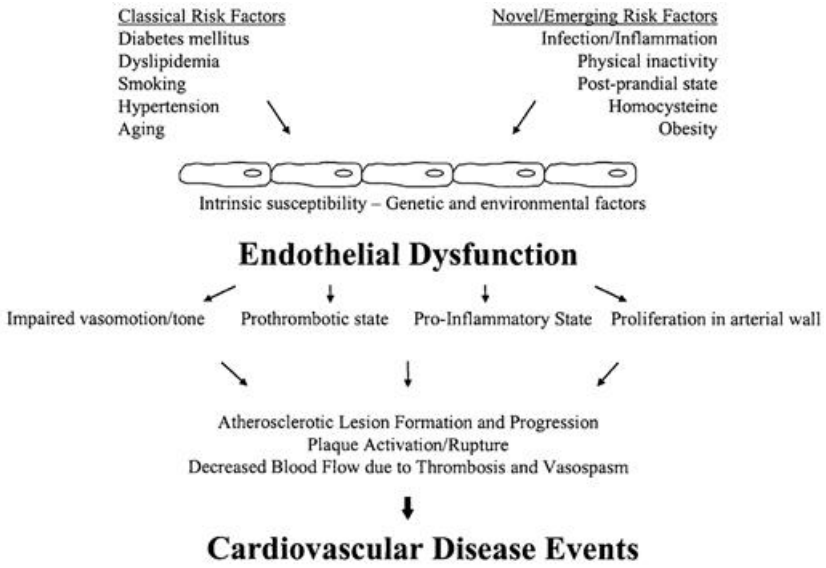


Figure 4: Factors inducing endothelial dysfunction and its role in the pathogenesis of cardiovascular disease events

ADMA is an endogenous competitive NOS inhibitor that can be metabolized by dimethylarginine dimethylaminohydrolase (DDAH) or excreted by the kidneys. ADMA is increased in patients with increased CV risk [126] and predicts future CV events [127]. Accumulation of ADMA in CKD could promote atherosclerosis and is related to macrovascular disease in patients with type 1 or type 2 DM and macroalbuminuria [128]. In type 2 DM patients with albuminuria, increased ADMA was also linked to declining GFR and subclinical inflammation, as assessed using high sensitivity C-reactive protein (hsCRP) [129].

In addition to its direct inhibitory effect on endothelial NOS, ADMA may cause endothelial dysfunction via other mechanisms. These include increased superoxide production by activation of NADPH-oxidase (nicotinamide adenine dinucleotide phosphate-oxidase), thereby interfering with NO bioavailability; activation the local RAAS, thus contributing to the development of arteriolar dysfunction and increased tone; and a decrease in the number of circulating endothelial progenitor cells (EPCs), possibly interfering with vascular repair mechanisms [130].

Insulin resistance also plays an important role in endothelial dysfunction. Insulin resistance has been associated with renal disease in both types 1 [131] and type 2 [132] DM, thereby offering another possible explanation of the association between CV disease and DKD.

Endothelial cell injury has recently been linked to increased shear stress [133]. The latter is known to be associated with high BP.

Plasma levels of the soluble RAGE are strongly associated with CVD in patients with DKD [134]. RAGE induces the production of adhesion molecules and inflammatory cytokines, thus promoting endothelial and renal dysfunction, low grade inflammation and vascular remodeling. Through these mechanisms, RAGE may contribute to both DKD and CV disease.

Elevated levels of uric acid have been associated with inflammation, oxidative stress, insulin resistance, dysglycemia, endothelial dysfunction, vascular stiffness, cardiac diastolic dysfunction, renal hyperfiltration and proteinuria [135]. Uric acid decreases NO production [136]. Experimental hyperuricemia causes afferent renal arteriolopathy and tubulo-interstitial fibrosis in the kidney by activating the RAAS [137]. Uric acid also activates cytoplasmic phospholipase A2 and NF-əB [138] and increases inflammation [139]. Lowering uric acid reduces tubulointerstitial fibrosis both in the 5/6th nephrectomised model [140] and in diabetic nephropathy [141]. Additionally, withdrawing uric acidð lowering therapy increases urinary transforming growth factor beta 1 (TGFɓ-1) in hyperuricemic patients with CKD [142]. The putative mechanisms by which increased serum uric acid may contribute to CKD onset and progression are illustrated in (Figure 5) [135].

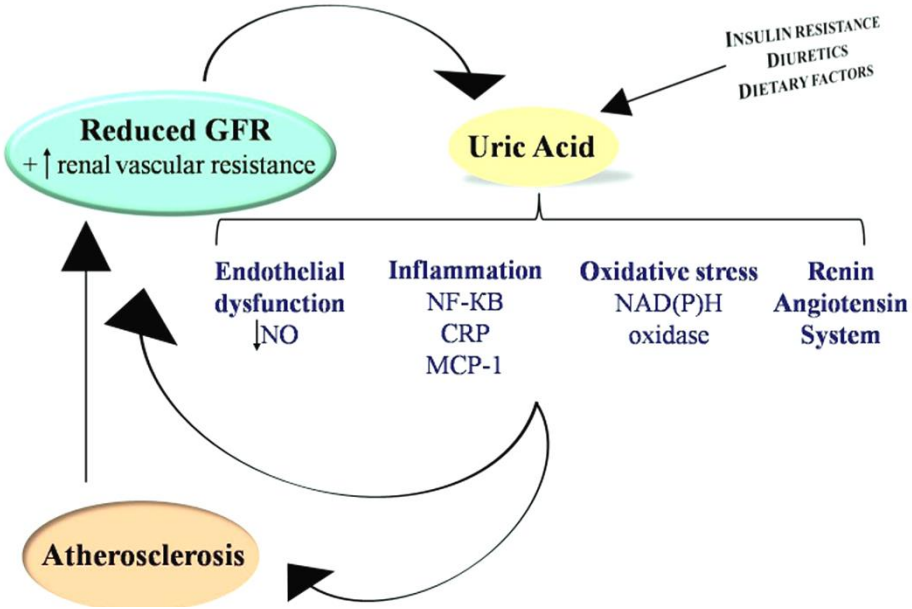


Figure 5: Putative mechanisms by which elevated serum uric acid level may contribute to CKD development, progression and atherosclerosis

Activation of the RAAS in DKD results in endothelial dysfunction. When angiotensin II activates the AT1 receptor, it stimulates the generation of reactive oxygen species by NAD(P)H oxidase and other enzyme systems, leading to upregulation of inflammatory mediators, which include cytokines, chemokines, adhesion molecules, and plasminogen activator inhibitor 1, and superoxide scavenging of NO. These events promote endothelial dysfunction, vascular remodeling and the progression of atherosclerosis [143].

Hyperhomocysteinemia may also play a role in endothelial dysfunction. Elevated plasma homocysteine levels are associated with albuminuria in both type 1 [144] and type 2 DM [145]. The increased susceptibility of diabetic subjects to hyperhomocysteinemia may be secondary to an acceleration of glucose-induced oxidative stress on endothelial cells [144]. This hypothesis is supported by animal studies showing that homocysteine-induced endothelial dysfunction occurs much more readily in the presence of diabetes than in its absence [146]. However, in clinical trials treatment of hyperhomocysteinemia did not improve outcomes.

Endothelial progenitor cells (EPCs): Bone marrow-derived EPCs in peripheral circulation contribute to re-endothelialization of injured vessels as well as to neovascularization of ischemic lesions and therefore play a key role in maintaining the integrity of the vascular system. Various factors influence the number and function of EPCs. Mild-to-moderate renal dysfunction accompanying stable angina has been shown to be associated with EPC depletion, irrespective of angiographic coronary artery disease extent. This may exacerbate an imbalance between endothelial injury and EPC-mediated repair, thus contributing to high CV risk in coronary artery disease coexisting with renal insufficiency. In addition, the number of EPCs is decreased in both patients with type 1 and type 2 DM [130].

Inflammation: CKD results in a chronic, low-grade inflammatory process that becomes evident even in the early stages of the disease. Circulating levels of inflammatory markers, such as CRP and interleukin-6 (IL-6), are elevated in CKD patients. There is a relationship between inflammatory activation of peripheral blood mononuclear cells and UACR in type 2 DM. Increased inflammation could thus play a role in accelerated atherosclerosis and increased CV risk associated with DKD [147].

Advanced glycosylation end-products (AGEs): AGEs are products of non-enzymatic glycation and oxidation of proteins and lipids that are increased in situations with hyperglycemia and oxidative stress such as DM. The kidney plays an important role in clearance and metabolism of AGEs. In CKD, AGE concentrations increase, partly by an increase in oxidative and carbonyl stress. AGEs and their receptors are thought to play an important role in the pathophysiology of DKD and vascular injury [148, 149].

Lipoprotein abnormalities: Lipoprotein abnormalities also play a role. DKD is characterized by low high-density lipoprotein (HDL)-concentrations and elevated intermediate-density lipoprotein (IDL), both of which contribute to an increased CV risk. Elevated serum creatinine has a greater impact than albuminuria on abnormalities in IDL and HDL [150]. In addition, in diabetic patients, serum lipoprotein (a) concentration is associated with albuminuria, further contributing to the elevated CV risk [151].

Adhesion molecules: Elevated plasma concentrations of soluble adhesion molecule concentrations in patients with DKD could be markers of atherosclerosis and increased CV risk. Thus, plasma concentration of soluble intercellular adhesion molecule-1 (sICAM-1) is elevated in type 1 DM patients with microalbuminuria and also the concentrations of sICAM-1 are elevated in patients with macroalbuminuria and normal serum creatinine [152].

Hyperfibrinogenaemia: Hyperfibrinogenaemia is a CV risk factor. Fibrinogen was higher in albuminuric type 2 DM subjects than in type 2 DM normoalbuminuric patients [153].

Cardiac autonomic neuropathy: In type 2 DM patients, cardiac autonomic neuropathy and DKD were independently associated with asymptomatic coronary artery disease [154]. In type 1 DM patients with nephropathy, cardiac autonomic neuropathy assessed as heart-rate variation during deep breathing was an independent risk factor for CV morbidity and mortality [155]. In addition, autonomic neuropathy may be associated with increased central arterial stiffness [156].The mechanisms of increase CV morbidity and mortality by cardiac autonomic neuropathy are still under debate. Suggested mechanisms include impaired central control of respiration in patients with cardiac autonomic neuropathy [157]; exercise intolerance with a reduced response in heart rate and BP and decreased cardiac output during exercise; and possibly QT prolongation [158, 159].

Other possible links between cardiovascular disease and DKD

Several additional hypotheses have been proposed to explain the link between the CKD and CVD [122]. Local factors may play a role in the cross-talk between the kidney and the CV system. It was hypothesized that enzymes involved in the proteoglycan metabolism are the primary cause of albuminuria and the associated complications [160]. However, there is little evidence supporting this hypothesis. An alternative hypothesis is a common glycocalyx defect in the glomerulus and in the systemic circulation. The endothelial glycocalyx plays a significant role in the genesis of vascular disease and controls vascular permeability [161, 162].

Accumulation of toxins may also link reduced renal function and endothelial dysfunction [122]. This may be a consequence of accumulation of the toxins that are normally excreted via the kidneys or the reduced kidney dependent catabolism of molecules such as ADMA or catecholamines.

Systemic factors may also play a role; such as enhanced sympathetic activity at an early stage of renal disease when whole-kidney GFR is still normal. Increased sympathetic afferent signals originating from the damaged kidney may contribute to increased incidence of sudden death and mortality secondary to ischemia in renal patients. However, the triggering intrarenal mechanisms have not yet been identified [122].

Genetic influences probably also play a role. The P12A and C161T polymorphisms of the peroxisome proliferator activated receptor gamma (PPAR-ɔ) gene are important predictors of CVD in patients with DKD [163]. Another interesting polymorphism is the leucine 7 to proline 7 (Leu7Pro), located in the signal peptide of the human preproneuropeptide Y [164]. In the Finnish Diabetic Nephropathy (FinnDiane) Study, the Leu7Pro substitution was independently associated with HbA1c, proteinuria, and coronary heart disease in multivariate analysis, suggesting that it may contribute to the genetic susceptibility to DKD and CVD in type 1 DM patients.

Short stature is associated with increased mortality in diabetic and non-diabetic subjects with ESRD [165]. Short stature has been linked to nephropathy in both type 1 [166] and type 2 DM [167]. This may be due to common genetic and/or environmental factors predisposing to short stature, DKD and CVD. In addition to genetic influences, the hypothesis of ñnephron underdosingò suggests that aberrant fetal programming secondary to genetic factors, malnutrition, and other insults in utero leads to the formation of fewer glomeruli [168]. Future ESRD is predicted by baseline BP [169]. Congenital variability in filtration surface area may contribute to the varying susceptibility of patients exposed to potentially injurious renal stimuli to eventually manifest chronic nephropathy, as well as the different susceptibility of subsets of type 1 and type 2 DM to develop overt glomerulopathy. Kidney transplantation in 5/6 nephrectomized animals protects against albuminuria and rise in BP [170]. In humans, single kidney GFR declines more rapidly in uninephric individuals compared to binephric ones [171]. The role of fetal programming on the occurrence of renal and CVD needs further study.

4. Assessment of cardiovascular risk in Diabetic CKD

The analysis of the plasma levels of novel mediators in DKD patients may provide insights into pathogenesis and eventually facilitate tools for risk stratification and for the design of novel therapeutic strategies. In this regard, novel prognostic biomarkers of mortality in CKD and ESRD have allowed the identification of potential key processes in CVD mortality [172]. Although simplified, this approach helps to build a conceptual framework of the pathogenesis of CVD in CKD and understand the interconnections between biological processes [173]. We have chosen a representative of inflammatory mediators, Chemokine (C-X-C Motif) Ligand 16 (CXCL16) and another from the regulators of lipid metabolism, Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9), for further studies in DKD patients.

Chemokine (C-X-C Motif) Ligand 16 (CXCL16)

Chemokines are divided into four major subfamilies, C, CC, CXC, and CX3C, based on the number and spacing of conserved cysteines. Biological activities of chemokines have been most clearly defined in leukocytes, where chemokines coordinate development, differentiation, anatomic distribution, trafficking, and effector functions and thereby regulate innate and adaptive immune responses [174]. CXCL16 belongs to the CXC chemokine family.

CXCL16 is a chemokine and cell membrane receptor

CXCL16 is synthesized as a transmembrane molecule [175]. CXCL16 has an atypical structure, being larger (254 amino acids) than other chemokines, and being composed of an extracellular N-terminal

chemokine domain, a glycosylated mucin-like stalk, a transmembrane domain, and a short cytoplasmic tail containing a potential tyrosine phosphorylation site that may bind SH2 [176, 177]. The presence of a transmembrane domain is unusual for a chemokine, and allows CXCL16 to be expressed as a cell surface bound molecule, as well as a soluble chemokine [178]. CXCL16 interacts with the CXC-chemokine receptor CXCR6, also known as Bonzo [179]. CXCL16 was originally described as a scavenger receptor for phosphatidylserine and oxidized low-density lipoprotein (oxLDL) and termed SR-PSOX [180]. Expression of CXCL16 is induced by the inflammatory cytokines; interferon gamma (IFNɔ) and tumor necrosis factor alpha (TNFα) [179]. CXCL16 has been implicated in the pathogenesis of vascular, kidney and lung injury [181, 182].

Upon cleavage of the mucin-like stalk, the CXCL16 chemokine is released and functions as a chemoattractant for CXCR6+ T, NK, NKT (Natural Killer T cell), B, and dendritic cells [176, 177, 183, 184]. Cleavage is mediated by A Disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), a member of the disintegrin and metalloproteinase domain family of enzymes [185, 186]. Because CXCL16 functions as a chemokine, a scavenger receptor, and an adhesion molecule, it may be involved in several phases of the immune response from antigen recognition to migration of immune cells into inflammatory foci, including atherosclerotic plaque [187].

CXCL16 as a new biomarker of kidney injury

While much is known from animal-based studies, little is known about CXCL16 in human subjects. Urinary CXCL16 levels are significantly higher in active systemic lupus erythematosis (SLE) patients with renal disease than in active SLE patients without renal disease [179]. Serum CXCL16 levels were significantly increased in CKD and gout subjects and were independently associated with a change of renal function [188, 189]. Taken together, these findings suggest that CXCL16 may be a marker of renal disease. No significant differences in serum CXCL16 levels between healthy and type 2 DM subjects were found. However, CXCL16 levels were higher in DKD patients than in CKD patients without diabetes [189]. CXCL16 levels were independently associated with renal function in DKD and not with diabetes itself. Circulating CXCL16 levels were negatively correlated with eGFR and blood albumin, and positively correlated with creatinine and proteinuria in DKD subjects after adjustment for age, gender and BMI suggesting that elevated CXCL16 levels are closely related to glomerular injury and declining renal function in DKD patients [190].

However, the mechanism responsible for the elevation of CXCL16 concentration and its role in the pathophysiology of DKD is not fully understood. CXCL16 expression in subjects with DKD may be related to the abnormalities of cholesterol metabolism. Thus, elevation of CXCL16 was associated with higher levels of oxLDL were found in streptozotocin-induced diabetic mice [191], increased glomerular CXCL16 expression was also accompanied by high levels of oxLDL in patients with glomerular kidney diseases [192].

CXCL16 plays a major role in the uptake of oxLDL by podocytes but not by mesangial and tubular renal cells. Abnormalities of podocyte structure and function play a major role in the onset of albuminuria both in diabetic and non-diabetic nephropathy [193]. The podocyte dysfunction-oxLDL-CXCL16 axis may play a role in DKD.

CXCL16 as a biomarker for CV risk

Atherogenesis is thought to involve inflammatory responses to oxLDL and its free oxidized lipid constituents, which accumulate in the arterial intima [194]. These responses include local release of chemokines that collaborate with cytokines and adhesion molecules to promote infiltration of leukocytes into vascular subendothelium [195, 196]. In contrast to this paradigm, genetic evidence has suggested that the chemokine CXCL16 may have atheroprotective factor actions [182], through unknown mechanisms that may be related to its scavenger receptor functions.

CXCL16 is undetectable in normal aorta, but is expressed in mouse and human coronary and carotid atherosclerotic lesions, colocalizing with lipid-laden intimal macrophages and smooth muscle cells [187, 197]. Studies evaluating soluble CXCL16 as a potential biomarker of coronary artery disease have been inconsistent [198, 199]. A polymorphic variant of CXCL16 named CXCL16-A181V was associated with increased coronary artery stenosis in post-infarction patients [200]. Genetic inactivation of CXCL16 in hyperlipidemic LDL receptor (LDLR) knockout (KO) mice (ldlr-/-) results in accelerated atherosclerosis due to enhanced macrophage recruitment to the aortic arch [182]. Although the CXCL16-CXCR6 relationship is considered monogamous, CXCR6 inactivation in atherosclerosis-prone apolipoprotein E (ApoE) KO mice (apoE-/-) decreased susceptibility to atherosclerosis, and was accompanied by reduced homing of lymphocytes into the aorta. These data suggest that CXCR6 is a proatherogenic chemokine receptor [201].

Lipid-induced down-regulation of surface ADAM10 favors the scavenger role of cell surface CXCL16. However, the mechanism through which atherogenic lipids down-regulate ADAM10 remains unclear. Thus, although CXCL16 exists in two different forms, soluble and membrane bound, the atherogenic environment may dictate preference of a single configuration that may determine the role of CXCL16 in atherosclerotic lesions. When expressed in a membrane-tethered form, CXCL16 could conceivably support cell-cell adhesion [202], which could play a role in plaque evolution. Consistent with this, transmembrane CXCL16 functions as a proadhesive chemokine promoting adhesion of primary cells, predominantly T cells to fibroblastic reticular cells in the lymph node [203], to the follicle-associated epithelial cells covering Peyerôs patches [204] and to the vascular endothelium [205], suggesting that the CXCR6-CXCL16 axis may contribute critically in the formation of primary and secondary T cell responses.

Cellular cholesterol efflux constitutes a potent physiological protective system against atherosclerosis [206, 207]; however, the involvement of many players in the metabolic pathway of HDL makes this system a difficult therapeutic target. A link between CXCL16, HDL, and macrophage cholesterol efflux has been established, and provided the first experimental support at the molecular and cellular level using primary human cells relevant to atherosclerotic plaque for the scavenger mechanism of atheroprotection mediated by CXCL16 [208].

The ability to reflect upstream inflammatory activity is an important criterion for an ideal biomarker in CVD. Because of the link between several inflammatory mediators and the cleavage of the membrane-bound to the soluble form of CXCL16, it has been suggested that soluble CXCL16 could serve as a reliable marker of inflammation. The high stability of CXCL16 (eg, little circadian variation, minor influence of food intake, and little variation after freeze and thaw cycle) further support a potential role of CXCL16 as a biomarker in clinical samples [209].

Onset of acute coronary syndrome appears to involve the rupture or erosion of atheromatous plaques with abundant infiltration of inflammatory cells, such as monocytes/macrophages and T-lymphocytes. In addition, OxLDL appears to play a role in the formation of erosion- or rupture-prone vulnerable plaques by enlarging the lipid core, thinning of the fibrous cap, and evoking proinflammatory responses, which result from foam cell transformation of macrophages, apoptosis of vascular endothelial and smooth muscle cells and macrophages, production and activation of matrix metalloproteinases and induced expression of chemokines and endothelial-leukocyte adhesion molecules [210]. The association between circulating CXCL16 levels obtained within 24 hours after admission and time to death was assessed in 1351 patients with a diagnosis of unstable angina, non ST-segment elevation myocardial infarction, or ST-segment elevation myocardial infarction. During a median follow-up time of 81 months, high CXCL16 levels were prognostically unfavorable. After adjustment for conventional risk markers, logarithmically transformed CXCL16 level remained a strong independent indicator of long-term mortality (hazard ratio 1.21; 95% CI 1.09 to 1.36 per 1 SD increase in CXCL16; P<0.01) and congestive heart failure development (hazard ratio 1.25; 95% CI 1.05 to 1.48; p=0.01). In a subsample of 714 patients, after further adjustment for troponin T, hsCRP, proï B-type natriuretic peptide, and LV ejection fraction, CXCL16 still provided significant additional prognostic information on mortality (hazard ratio 1.21; 95% CI 1.02 to 1.42 per 1 SD increase in CXCL16; p=0.02) [209]. These findings support a pro-atherogenic effect of CXCL16 in human coronary artery disease [209]. Increased circulating chemokines, including CXCL16 have been reported during acute myocardial infarction and might give prognostic information about future ischemic events [211]. However decreased or increased CXCL16 levels in both stable and unstable angina was reported in different studies [199, 212, 213]. Soluble CXCL16 induces inflammatory responses in vascular smooth muscle cells and peripheral-blood mononuclear cells. With relevance to plaque destabilization, soluble CXCL16 enhances matrix metalloproteinase activity [198, 214]. Also, CXCL16 may have constitutive functions, such as promotion of cell survival and normal leukocyte recruitment. Thus although too much CXCL16 may be harmful, too little may not necessarily be beneficial.

Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9)

PCSK9 was first described in 2003 as neural apoptosis-regulated convertase-1 [215] and was renamed PCSK9 after it was found to be a member of the PCSK family of pre-protein convertases [216].

PCSK9 function

PCSK9 is the third gene [in addition to LDLR and apolipoprotein B (ApoB)] to cause autosomal dominant hypercholesterolemia [215]. Autosomal dominant PCSK9 activating mutations account for 1% of cases of familial hypercholesterolemia. Single nucleotide polymorphisms (SNPs) or mutations are associated with asymptomatic slightly reduced LDL-cholesterol levels, but only one patient with

homozygous PCSK9 deficiency has been described. This patient is hypocholesterolemic, with a lipoprotein phenotype of hypobetalipoproteinemia, and is reportedly fit and well [217]. A patient with a dominant negative phenotype and hypobetalipoproteinaemia has also been described [218] although most mutations show co-dominant (additive) effects.

PCSK9 is a 692 amino acids protein, which regulates LDLR expression [219]. LDLR represents the main route of elimination of PCSK9 and a reciprocal regulation controls serum PCSK9 levels, hepatic LDLR expression, and serum LDL levels [220]. PCSK9 subcellular expression profile follows that of the LDLR [219]. Thus, PCSK9 is highly expressed in hepatocytes, enterocytes, brain [216] and in other cells including macrophages [216, 221]. PCSK9 has a highly self-specific auto-catalytic protease domain involved in degradation of the LDLR [216, 221] though this is not required to reduce LDLR expression [222, 223]. In general, autosomal dominant activating mutations in PCSK9 cause familial hypercholesterolemia by reducing LDLR expression. By contrast, inactivating mutations upregulate LDLR expression and are associated with reduced plasma LDL-cholesterol. Two inactivating stop mutations common in African-Americans account for the lower LDL-cholesterol levels found in this group [224]. In the Atherosclerosis Risk in Communities (ARIC) study, the YI42X mutation present in 0.8% or the C679X mutation present in 1.8% resulted in 28% lower LDL-cholesterol and reduced risk of CVD from 9.7 to 1.2%, that is, by 88% over the 15-year period of follow up [224]. However, while most inactivating PCSK9 mutations are co-dominant (i.e., additive in effect), a few are dominant [218]. PCSK9 contributes to cholesterol levels in familial hypercholesterolemia. Patients with familial hypercholesterolemia with lower untreated LDL-cholesterol had lower PCSK9 levels (152 ng/ml) compared with unaffected controls (177 ng/ml) and also patients with high LDL-cholesterol familial hypercholesterolemia (186 ng/ml) [225]. Consistent with these findings, subjects carrying loss-of-function mutations (Y142X and C679X) exhibited a 28% reduction of plasma LDL-cholesterol levels and an 88% decrease in the risk of coronary heart disease in a 15-year follow-up survey [224]. The human genetic data are in agreement with the observations in mice. Plasma cholesterol levels are approximately 50% lower in PCSK9 KO mice owing to increased clearance of lipoproteins from plasma [226]. No apparent physiological or behavioral abnormality was observed in KO mice or humans carrying compound heterozygous loss-of-function mutations [226, 227]. Mice with PCSK9 deficiency are hypocholesterolemic, hyperglycemic, hypoinsulinaemic and had abnormal pancreatic islets [228] but this full phenotype does not seem to occur in man.

PCSK9 activity is related to fasting, postprandial lipid metabolism [229] and affected by insulin regulation of the sterol regulatory element binding protein 1c (SREBP-1c) [230]. PCSK9 is secreted by enterocytes in the ileum and by hepatocytes in plasma in response to eating [231, 232]. One hypothesis is that PCSK9 transiently down regulates LDLR on the liver so that triglyceride-rich lipoproteins are not subject to first pass clearance and metabolism but are allowed to enter the general circulation. Consequently, triglyceride-rich lipoproteins are available for the action of lipoprotein lipase to release free fatty acids for uptake by muscle and adipose tissue. Once circulating PCSK9 has been cleared, hepatic LDLR is upregulated and triglyceride-rich lipoproteins and later LDL are cleared from the circulation. This action of PCSK9 is not the only mechanism that downregulates LDLR as similar actions have been shown for the Idol protein that acts in a reciprocal manner to PCSK9 and can be affected by statins and is controlled by liver-X receptor (LXR)-mediated pathways [233]. The extracellular pathway of PCSK9 to regulation of LDLR function oversimplifies the actual regulatory contribution. In addition, intracellular PCSK9 is present both in the liver and in enterocytes and also regulates local LDLR expression, yet the function and control of this pathway and how it relates to the extracellular pathway are unclear [234]. PCSK9 may also directly affect atherogenesis. PCSK9 secreted after overexpression by smooth muscle cells, upregulates macrophage LDLR expression [221]. Cross-breeding experiments in mice have investigated the role of PCSK9 in atherosclerosis [235]. These involved mating C57BL/6 wild-type (WT), apoE-deficient and LDLR-deficient mice with PCSK9 deficient, WT or overexpressing mice. Following 12-months Western diet PCSK9 KO mice accumulated fourfold less aortic cholesterylesters than WT mice, whereas PCSK9 overexpressing mice exhibited high aortic cholesterylesters and severe aortic lesions. The experiment was repeated in apoE deficient mice. After 6 months of regular diet, PCSK9 KO/ApoE KO mice showed a 39% reduction in atherosclerosis and aortic cholesterylesters accumulation compared with PCSK9 WT/ ApoE KO mice, while PCSK9 overexpressing/ApoE KO mice showed a 137% increase. No influence of PCSK9 expression was observed in LDLR KO mice, suggesting that PCSK9 modulates atherosclerosis mainly via the LDLR.

Commercially available enzyme-linked immunosorbent assays (ELISA) have been used to better understand PCSK9 function and biomarker potential, while activity assays are complex [236]. In cross-sectional studies associations of PCSK9 mass and activity have been described with levels of androgens, estrogens, insulin and growth hormone [229, 237] but other as yet unstudied hormones may affect PCSK9 activity. Sex steroids can induce changes in PCSK9 activity, and these are suspected to be responsible for

the increase in LDL-cholesterol during puberty in boys and following the menopause in women [229, 237]. PCSK9 shows a diurnal activity that parallels cholesterol synthesis as measured by lathosterol levels, and this rhythm is abolished by statin or bile acid sequestrant therapy [238].

PCSK9 and the Kidney

Heavy glomerular proteinuria, a hallmark of nephrotic syndrome, is associated with severe hyperlipidemia and lipiduria. Hypercholesterolemia, increased plasma LDL, impaired LDL and HDL clearance, and depressed maturation of HDL are common features of dyslipidemia in nephrotic syndrome [239]. These abnormalities are due to acquired hepatic LDLR and HDL docking receptor (SRB1; scavenger receptor class B1) deficiencies as well as urinary excretion and reduced plasma concentration and enzymatic activity of lecithin cholesterol acyltransferase (LCAT) [240]. In addition plasma concentrations of triglycerides, very low-density lipoprotein (VLDL), and IDL are increased, and triglyceride content of various lipoproteins is elevated [241]. Nephrotic syndrome results in impaired clearance of triglyceride-rich lipoproteins, VLDL, chylomicrons, and their remnants [239]. The latter is caused by down-regulations of the primary pathways of triglyceride-rich lipoprotein clearance including lipoprotein lipase [242] and VLDL receptor [243] in the muscle and adipose tissues and of hepatic triglyceride lipase [244]. In addition, increased hepatic production of fatty acids and triglycerides has been demonstrated in various models of nephrotic syndrome [245].

Plasma PCSK9 concentration was higher in patients with nephrotic syndrome (n=15, 15±5 ng/mL) and peritoneal dialysis (13±1 ng/mL) than in healthy controls (9±0.6 ng/mL) and hemodialysis (7.0±0.5 ng/mL) patients. Plasma PCSK9 level was directly related to total and LDL cholesterol concentrations in the study population. Higher plasma PCSK9 concentration may contribute to elevation of LDL levels by promoting LDLR deficiency in nephrotic syndrome and peritoneal dialysis [246]. Upregulation of PCSK9 and inducible degrader of the LDL receptor (IDOL) was also observed in experimental nephrotic syndrome [247].

In another study serum PCSK9, total cholesterol, LDL-cholesterol and HDL-cholesterol levels were significantly lower in hemodialysis than in control individuals. In hemodialysis patients not on statins PCSK9, total cholesterol and LDL- cholesterol levels were lower than in control subjects and PCSK9 levels correlated with total cholesterol, LDL-cholesterol and triglyceride levels. In hemodialysis patients on statins PCSK9 levels were not significantly different from the non-CKD group and did not correlate with total or LDL-cholesterol levels. These data suggest that the regulation of LDL-cholesterol by PCSK9 remains intact in hemodialysis patients [248].

PCSK9 and cardiovascular risk

Dyslipidemia is one of the most significant risk factors for CVD, accounting for 50% of the population-attributable risk for myocardial infarction [249] and 25% of the population-attributable risk for stroke [250]. The effective management of dyslipidemia is therefore essential for the reduction of CV risk.

Nonsense PCSK9 mutations are associated with a 20-40% reduction in mean LDL cholesterol [251, 252]. In the ARIC study during a 15-year-follow-up period, the 10 percent of black subjects who did not have a nonsense PCSK9 mutation experienced a coronary event. In contrast, only 1% of subjects with a non-sense mutation of PCSK9 developed CVD [224]. The calculated hazard ratio for coronary heart disease among carriers as compared with noncarriers, after adjustment for age and sex, was 0.11. The incidence was still significantly low also after taking into consideration the BP and diabetes.

Autosomal dominant hypercholesterolemia patients with gain of function PCSK9 mutations had significantly higher serum total cholesterol concentrations and appeared to be more resistant to statin therapy compared to heterozygous familial hypercholesterolemia patients with known mutations in LDLR. Ischemic heart disease occurred earlier in patients with PCSK9 mutations than in patients with LDLR mutations. The reason underlying this severe phenotype associated with the PCSK9 $D^{374}Y$ mutation is not yet understood and has been suggested to be related to a possible positive role of PCSK9 on apoB secretion [253]. PCSK9 increases apoB secretion is independent of the LDLR. PCSK9 binding to apoB may protect apoB from the intracellular degradation [254].

Thus, putative gain or loss of function mutants correlate with increased or reduced plasma LDL levels and CV risk, respectively. A genome-wide association study observed a linkage between a SNP at a locus near PCSK9 with early-onset myocardial infarction [255].

Accordingly, PCSK9 could represent a safe and effective pharmacological target to increase clearance of LDL cholesterol and to reduce the risk of coronary heart disease. Recent clinical trials showed that anti-PCSK9 monoclonal antibodies that block the interaction between PCSK9 and LDLR and inhibit the PCSK9-mediated LDLR degradation considerably reduce LDL-C levels by up to 65% when given alone and by up to 72% in patients already receiving statin therapy [256]. PCSK9 binding to LDLR

can be blocked by >80% using anti-PCSK9 polyclonal antibodies [257]. Different clinical trials in phase I-II which are studying new lipid-lowering therapies based on PCSK9-neutralizing antibodies [258-262].

PCSK9 and Experimental Atherosclerosis and Diabetes Mellitus

Although the regulatory pathways and enzymes involved in cholesterol homeostasis in mice are dramatically different than humans, mice studies have provided some relevant information [221]. PCSK9 null and transgenic mice crossed with LDLR-/-mice show similar plasma cholesterol levels and plaque size compared to LDLR$^{+/+}$ mice. These data indicated that the effect of PCSK9 on cholesterol homeostasis and atherosclerotic plaque development is almost entirely dependent by the presence of LDLR [263].

PCSK9 is present in human atherosclerotic plaques may locally regulate the expression of LDLR in macrophages [221]. Of interest, resistin, a small protein secreted by macrophages in humans and adipocytes in rodents [264], induces PCSK9 expression in hepatocytes and reduces LDLR levels [265], suggesting that resistin-mediated upregulation of PCSK9 may contribute to the dyslipidemia commonly observed in obesity and DM.

PCSK9 expression is also dependent by the action of insulin through the activation of SREBP-1c [230]. In diabetic rats PCSK9 expression was significantly decreased [266]. In hyperinsulinemic obese mice, PCSK9 was also decreased and has been shown to regulate LDLR, via the mammalian target of rapamycin complex 1 (mTORC1) kinase activity [267]. In the same model, the transcriptional factor hepatocyte nuclear factor 1Ŭ (HNF1Ŭ) regulated PCSK9 levels [267]. These data are consistent with previous work demonstrating that the HNF1 binding site adjacent to sterol responsive element (SRE) is the critical regulatory sequence motif, and HNF1Ŭ is the predominant working partner for SREBP-2 in the regulation of PCSK9 gene [268]. Taken together, PCSK9 expression appears to be deregulated under different pathological conditions such as DM and obesity, and is potentially involved in the associated dyslipidemia.

Assessment of cardiovascular risk using the pulse wave

Vascular stiffness increases left ventricular afterload and decreases coronary perfusion leading to CVD. Increased vascular stiffness, as measured by pulse wave velocity (PWV), is associated with increased CV and all-cause mortality in dialysis patients [269]. Indeed, arterial stiffness has been used as an end-point in interventional studies in CKD patients [270].

Aortic (Carotid Femoral) Pulse Wave Velocity (CF-PWV)

Large arteries are not simple tube conduction structures. They moderate systolic pressure increases and maintain sufficient diastolic level to guarantee myocardial perfusion. Under disease conditions these arteries lose their natural elasticity leading to high systolic and low diastolic BP levels, which determine high pulse pressure. Arterial stiffness is now considered an increasingly important biomarker in the evaluation of CV risk and the detection of incipient vascular damage associated with hypertension and/or atherosclerosis. Arterial stiffness is an independent predictor of CV mortality in the elderly, hypertensive, diabetics, and patients with CKD as well as in the general population [271-274]. As an example, a prospective, observational cohort study examined the impact of aortic stiffness on CV mortality among 265 ESRD patients on hemodialysis, including 50 diabetic patients. At baseline, the diabetic ESRD patients had significantly higher aortic PWV than the nondiabetic patients. During a mean follow-up period of 63 months, 81 deaths, including 36 CV deaths, were recorded. All-cause or CV mortality rates were higher in the diabetic as compared with the nondiabetic patients and also in those with higher aortic PWV than those with lower aortic PWV. In a multivariate model including 13 covariates, aortic PWV remained a significant predictor for CV and overall mortality but not for non-CV mortality. Thus, the increased aortic stiffness of the ESRD patients with DM was associated with higher all-cause and CV mortality rates [275]. The guidelines of the European Societies of Hypertension and Cardiology (2007-2009) have postulated arterial stiffness assessment, measurement of the carotid plaque and ankle brachial index (ABI) as markers of vascular status. Any alteration of these measurements may define a state of vasculopathy that significantly increases the evaluation of risk [276]. There are several methods to estimate arterial stiffness (Table 4) [277].

Table 4: Devices and methods used to determine arterial stiffness

		Mechanotransducer
Non-invasive	Pulse Wave Velocity	Tonometer
		Echotracking
		Doppler
	Local stiffness	Echotracking
		Magnetic resonance
	Systemic stiffness	Waveform shape analysis
Invasive	Aortic angiography	

Non-invasive assessment of arterial stiffness has been proposed for individual CV risk evaluation and early detection of vascular damage associated with hypertension and/or atherosclerosis [278, 279]. The CF-PWV has emerged as a gold standard due to its accuracy, reproducibility, relative easy measurement, and low costs. PWV has yielded prognostic value beyond and above traditional risk factors [279]. However, and in spite of its recognized value the evaluation of PWV in the clinical practice has been hampered among other factors by the absence of standardized methodologies of study and the lack of established normal/reference values for different populations [279, 280].

PWV assessment involves measuring the pulse wave transit time along the analyzed arterial segment and the distance between the pulse wave recording sites. Therefore, PWV values depend on both the path length measurement and the algorithm used to detect ñthe footò of the analyzed waves [272]. The algorithms most frequently used are the intersecting tangent algorithm and the point of maximal upstroke during systole. The pathway can be the direct distance measured between the carotid and femoral measurement sites (Figure 6) [277], the sternal notch-femoral measurement site distance, or the distance obtained by subtracting the carotid-sternal notch distance from the sternal notch-femoral distance (subtracted distance) (Figure 7) [271].

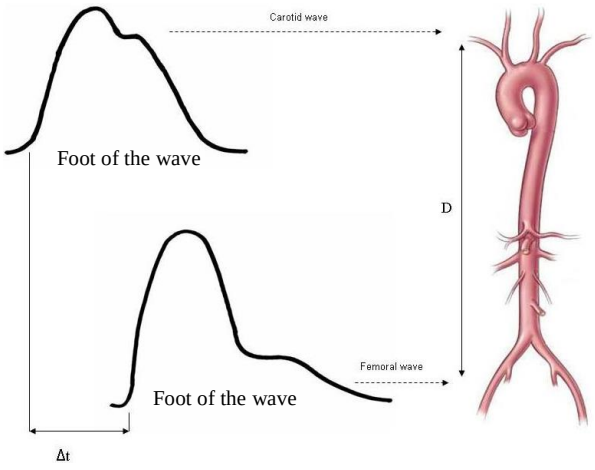
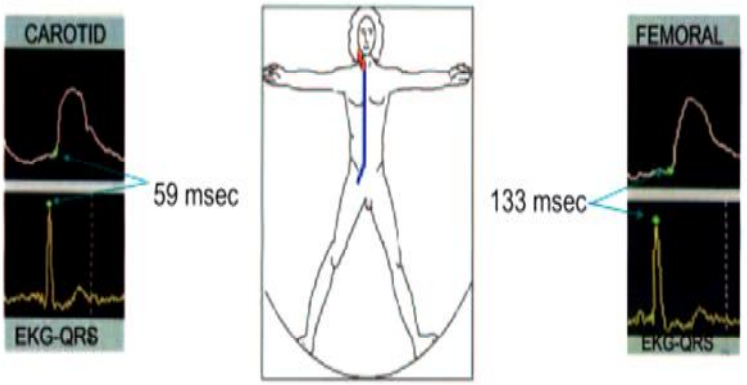


Figure 6: Pulse wave velocity determination. Transit time is estimated by the foot-to-foot method. The foot of the wave is defined at the end of diastole, when the steep rise of the waveform begins. The transit time (ϕt) is the time of travel of the foot of the wave over a known distance.



Velocity = Distance/Time

QRS-carotid	QRS-femoral	Δ time	Notch-carotid	Notch-femoral	Δ distance	Aortic PWV (Δdistance/Δtime)
59 msec	133 msec	74 msec	88 mm	696 mm	608 mm	8.2 m/sec

Figure 7: Measuring Aortic PWV. Elements necessary to measure PWV if done by tonometry coupled with electrocardiography (EKG). Distance from sternal notch to carotid pulse site (red) and distance from sternal notch to umbilicus and then to groin (in blue) are measured as shown. The probe captures the wave form at the carotid and femoral sites, and the time elapsed between the tip of the QRS complex and the onset, or ñfoot,ò of the pulse wav is calculated. Generally, 10 seconds worth of data are captured, yielding six to 11 beats, which are averaged for the elapsed time at the carotid and femoral sites. The distance divided by time generates the velocity. Because the distances are in millimeters and the times are in milliseconds, the ñmilliò cancels out, leaving units of m/s.

Different algorithms applied to the same waves can lead to differences in PWV of 5ï 15%, while differences in path length alone can result in differences in PWV of up to 30%. When determining normal and/or reference values those technicality-related issues should be considered. Arterial stiffness also depends on BP levels and increases with age [272, 279]. The potential impact of several factors on PWV is no longer observed after correction for age. Reference values for PWV have been recently provided from data mainly derived from European populations [279]. Given the ethnic diversity in CV risk profile, the dissimilar risk associations, and the differences in genetic-environmental interactions among populations, studies performed in a given population may not be directly applied to another different population. Then, considering the value of the vascular evaluation in CV risk stratification and diagnosis it is necessary to determine normal/reference levels for different vascular parameters taking into account the differences among populations [281].

The largest amount of evidence regards aortic stiffness, measured through CF-PWV. Aortic stiffness has independent predictive value for all-cause and CV mortality, CV disease, fatal and nonfatal coronary events and fatal strokes in patients with various levels of CV risk, including very high risk, as in ESRD [269, 275], high risk as in diabetes [282], medium risk (uncomplicated essential hypertension) [283] and low risk (general population) [284, 285] as well as in healthy elderly subjects [286, 287]. Aortic stiffness retains its predictive value for CV events after adjustment either to classical risk factors [286, 287]: brachial pulse pressure [288], Framingham risk score [288] or carotid intima-media thickness [286]. This indicates that aortic stiffness has a better predictive value than each of these factors [289].

PWV can be viewed mathematically or pragmatically.

From a mathematical point-of-view, the Moens-Korteweg equation relates PWV to a function of elastic modulus, viscosity, and vessel diameter, where V is wave speed, E is Young's elastic modulus in the circumferential direction (this modulus is a ratio of stress/strain, i.e., a way to represent the effects of force [stress] on shape [strain]), h is the wall thickness, ɟ is the density of the fluid, and R is the radius of the vessel [271].

$$V = \sqrt{(Eh / 2\rho R)}$$

From a pragmatical point-of-view, the velocity is simply how fast the pulse wave travels a specified distance of the vascular bed. This has been the clinical approach to measuring PWV in recent times. The steps involved in the actual technique are described later in this text.

Certain clinical conditions, such as advanced age, obesity, hypertension, DM, coronary artery disease, myocardial infarction, CKD and other factors unfavorably alter ventricular/vascular coupling, and impact on aortic PWV [290-294].

Genes: Aortic PWV is a heritable trait, according to Framingham data [295]. Several genetic polymorphisms have been reported to influence PWV, including those for the AT_1 receptor [296], fibrillin-1 [297], metalloproteinases [298], and the endothelin pathway [299].

Vessel Wall Proteins: Stiffness is also related to the relative amounts of elastin and collagen in the vessel wall. The more proximal parts of the aorta, which have slower velocities, contain relatively greater proportions of elastin compared with collagen. As one proceeds distally, this ratio changes [300]. Collagen also accumulates (relative to elastin) in the aorta with age and comorbidities such as hypertension, diabetes, and cigarette use. Excessive intramural build-up of other proteins, such as integrins, fibronectin, and desmin, also increase vascular stiffness [301].

Age: Age has one of the strongest effects on aortic PWV. Even in otherwise healthy individuals, aging results in an alteration of elastic wall components that generates an increase in stiffness[302]. Years of cyclic pulsatile stress lead to fragmentation of vascular elastin elements and accumulation of load-bearing collagen with a loss of stretch and increase in stiffness reflected by a steadily increasing systolic pressure [303].

Systolic and Pulse Pressures: These age-related changes enhance the magnitude of the pulsatile component of the stress and strain placed on the vessel with each heartbeat [304]. The level of BP creates an initial ñloadingò condition that regulates pressure wave conductance in the aorta. Increasing the BP acutely in a healthy individual will increase the PWV [305].

Calcification: Vascular calcification increases aortic stiffness [306]. Low bone turnover rates coupled with vitamin D and calcium salt therapies may be a ñperfect stormò setting for deposition of calcium in the aorta [307]. Aortic calcium loads as evidenced on lateral lumbar x-rays are directly related to aortic PWV [308].

Sodium Intake: Although salt intake may be naturally linked to an increase in systolic BP and thus stiffness, there are clear instances in models of hypertension in which aortic stiffness results from increased salt intake independent of BP changes [309].

Kidney Failure: Some of the most impressive data on CV outcomes in patients with stiff aortas have come from the ESRD population. Aortic PWV is increased in ESRD. Debate continues as to whether this is independent of shared risk factors. The search for specific mechanisms related to kidney failure apart from those covered already are areas of active investigation. Arterial stiffening has been linked to the rate of decline of kidney function, although this finding has not been replicated (Figure 8) [310].

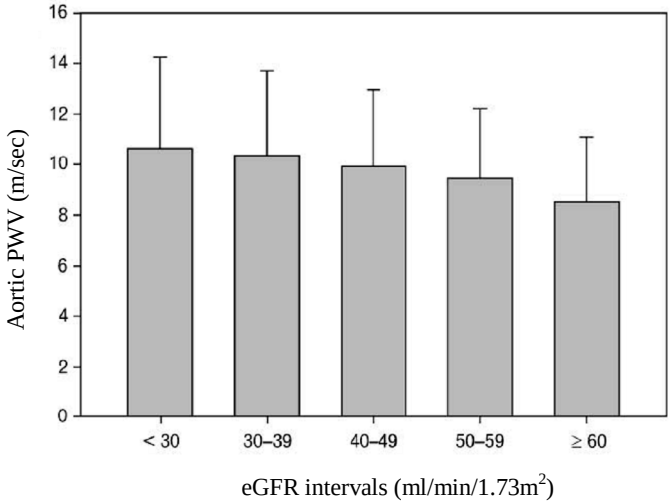


Figure 8: Aortic PWV and eGFR for 2564 participants in the CKD cohort study (P<0.001)

DM: Diabetes adds to the aortic PWV independent of other comorbidities or pathophysiologies, such systolic BP, kidney failure, and aging [275, 311]. Diabetes has long been thought of as a model of accelerated aging [312], and the build-up of inelastic matrix materials similar to that of aging in vessel walls and their subsequent glycation is a principal mechanism of the effects of diabetes on aortic PWV [313].

Aortic Pulse Wave Analysis (Ao PWA)

PWA uses a high-fidelity tonometer to capture electronically the shape of a peripheral arterial pulse. The tonometer probe is óapplanatedô (applied so as to flatten, but not occlude an artery) at a convenient site such as the radial artery. The shape of the peripheral pulse wave obtained is then calibrated with the brachial systolic and diastolic BP (measured in the traditional manner with an inflated cuff at the brachial artery) to derive the shape and dimensions of the central aortic pressure wave [314].

The SphygmoCor system converts the peripheral waveform to a central waveform using a proprietary algorithm (ógeneral transfer functions) [315, 316]. PWA permits the non-invasive measurement of three main indices of CV function: augmentation index adjusted to a heart rate of 75 beats per minute (AIX@75), subendocardial viability ratio (SEVR; or Buckberg index %), and the ejection duration index (ED %) [314]. The derivation of these indices is described below and shown schematically in (Figure 9) [317] and (Figure 10) [318].

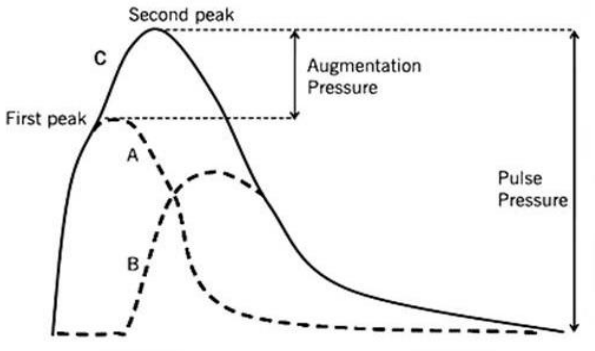
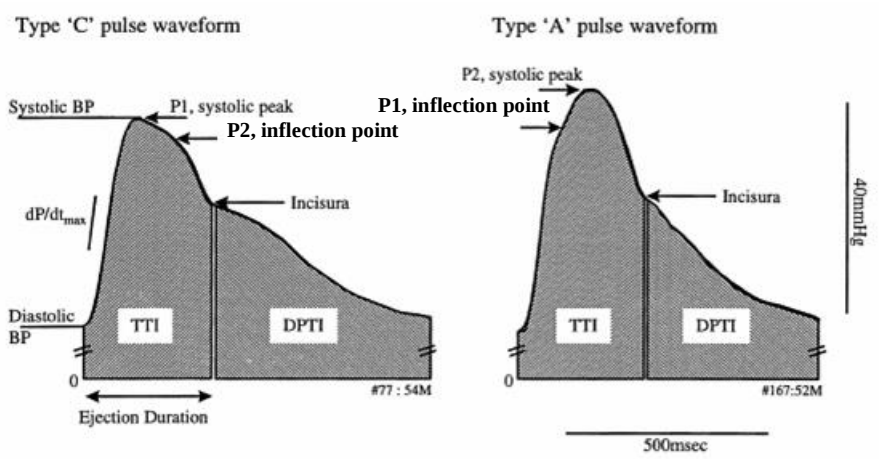


Figure 9: Central aortic waveform (C) is the result of the overlap between Forward waveform (A) and Reflected waveform (B). In this case the central aortic summation waveform is the result of early reflection in a patient with stiff arteries. Thus Augmentation Index = augmentation pressure / pulse pressure.



Augmentation Index (%) = (P2 ï P1) / PP

Subendocardial Viability Ratio (SEVR, Buckberg Index) (%) = DPTT / TTI

Figure 10: Types of the pulse waveforms. Type ñCò is defined by a dominant initial pressure peak with an inflection on the pressure waveform downstroke, whereas type ñAò is defined by a dominant late systolic peak and a shoulder with inflection on the upstroke. Type ñBò is intermediate, with no obvious inflection. Augmentation index (AIX) is calculated by the ratio of the difference between the pressure at the respective peaks or inflections (augmentation pressure, P2 ï P1) divided by the pulse pressure (Systolic BP ï Diastolic BP). Tension-time index (TTI) is the pressure-time integral during systole, and diastolic pressure-time index (DPTI) is the corresponding integral in diastole. Subendocardial viability is the ratio of DPTI to TTI. Schematic for dP/dtmax is shown.

Augmentation index (AIX) adjusted to a heart rate of 75 (AIX@75)

In the peripheral arteries the outgoing systolic pulse wave is reflected back towards the heart and adds to (ôaugmentsö) the central aortic pressure in late systole [315, 319, 320]. The amount by which the aortic pressure is increased by this phenomenon is the ôaugmentation pressureö. AIX is this aortic augmentation pressure expressed as a percentage of the aortic ôpulse pressureö [319, 320]. AIX (augmentation pressure/pulse pressure) indicates the combined influence of large artery PWV, peripheral pulse wave reflection and vascular function [320, 321]. AIX is the most widely researched index of PWA, with several studies indicating that AIX is independently predictive of adverse cardiac events [293, 322]. Since AIX varies with heart rate it is commonly adjusted to a ôstandard heart rateö of 75 beats per minute (AIX@75) [323].

The AIX is also associated with survival and it is a composite measure of the reflective properties of peripheral arteries and the elastic property of the large arteries [272]. Increased aortic stiffness is identified by an increase in the AIX and augmentation pressure.

Subendocardial viability ratio (SEVR; or Buckberg Index) %

The ôarea under the curveö (AUC) of the systolic and diastolic portions of the central aortic pulse wave can be measured using PWA. Blood flow within the coronary arteries occurs mainly during diastole and the diastolic-AUC indicates myocardial perfusion (ôsupply of oxygenö) [314, 324].The systolic-AUC indicates myocardial contraction (ôdemand for oxygenö). The subendocardial viability ratio (SEVR %), also known as the Buckberg index, is a ôsupply to demandö ratio of the diastolic-AUC divided by the systolic-AUC [314, 325]. The SEVR is a noninvasive estimate of myocardial perfusion relative to cardiac workload. Subendocardial ischemia occurs when SEVR% falls below 50% which is mainly due to reduction in diastolic perfusion times [326].

Ejection duration index (ED %)

The duration of LV systolic ejection; systolic time interval in milliseconds, can be measured using PWA. The ratio of the duration of systolic ejection to the total duration of a cardiac cycle is the ejection duration index (ED %). Patients with systolic dysfunction have a higher ED % than those with diastolic dysfunction [314].

Repeatability of PWA indices

It is important that clinical innovations, such as PWA, are carefully evaluated before their widespread introduction into routine clinical practice. The diffusion of previous medical innovations into medical practice has not always produced the benefits anticipated for patients [327]. For PWA to be clinically useful it must be a clinically reproducible technique [328]. Previous studies have demonstrated high levels of repeatability of AIX@75 and SEVR% [329, 330] and further studies have estimated the repeatability of SEVR% [331, 332], although one of these studies only addressed between-observer repeatability. No previous studies have reported on the repeatability of ED % [314]. Thus, we focused our study in AIX@75 and SEVR%.

There is an ample observational evidence of the involvement of inflammation as a determinant of outcomes in CKD. However, this evidence has translated neither into the adoption of novel biomarkers in routine clinical practice nor into specific therapeutic options or improved outcomes for the CKD population. Identification of key biomarkers as well as accumulating experience with biologicals targeting these biomarkers in non-CKD populations may provide the rational basis for novel therapeutic approaches to the dismal outcome of ESRD patients. The same situation regarding the application of the noninvasive screening methods of vascular stiffness for high CV risk patients in the daily clinical practice; looking for better quality of life and improving the long term outcome for those patients. We have started a prospective study assessing the impact of these novel biomarkers on outcomes and response to therapy. We now present the information gathered at the baseline visit in a cross-sectional analysis assessing predictors of biomarker levels and altered pulse wave parameters.

HYPOTHESIS AND OBJECTIVES

Hypothesis

The study of biomarkers and the pulse wave assessment will provide insights into the pathogenesis and monitoring of CVD in diabetic CKD patients that may eventually lead to improve outcomes.

Objectives

The general objective of this thesis is to characterize potential markers of vascular injury in patients with CKD which help understand the pathogenesis of vascular injury in CKD in order to develop novel therapeutic approaches, as well as to develop novel monitoring instruments.

1. To study the distribution of plasma values and the correlates of novel plasma biomarkers in diabetic non-dialysis CKD patients.

2. To study the prevalence and correlates of vascular injury as assessed by pulse wave assessment in hypertensive and CKD patients.

PATIENTS AND METHODS

The study was performed in Fundación Jiménez Díaz University Hospital ï Autonomous University of Madrid. The study was approved by the IIS-Fundacion Jimenez Diaz Ethics Committee. Before enrollment, the study was fully explained to all participating patients and an informed consent was signed.

Patients attending the CKD, diabetic nephropathy and hypertension clinics not on dialysis were offered to participate. No prior limits were provided for eGFR or albuminuria, although the nature of the clinics resulted in an overrepresentation of patients with some degree of albuminuria or decreased eGFR.

Exclusion criteria were: less than 18 years old, Hepatitis C Virus (HCV), Hepatitis B Virus surface Antigen (HbsAg) and Human Immunodeficiency Virus (HIV) positive serology patients, being on dialysis or transplantation or refusal to sign an informed consent.

Study design: This cross-sectional observational study assessed baseline data from the following two prospective cohorts of patients (Figure 11).

- A ñbiomarkerò cohort included 134 diabetic patients with CKD. In these patients the novel plasma biomarkers CXCL16 and PCSK9 were assessed. Additionally, biomarkers were assessed in 3 non-diabetic CKD patients and in 4 healthy controls.
- A ñPulse Wave Assessmentò cohort included 191 individuals. Of these, 153 were CKD patients and 38 non-CKD patients with hypertension or high cardiovascular risk. In these patients pulse wave assessment was performed.

Both cohorts had in common 54 CKD patients, for whom both Pulse Wave Assessment and biomarkers data were available.

Additionally, outside the main study, biomarkers were assessed in 3 non-diabetic CKD patients and in 4 healthy individuals. These patients didnôt belong to the analyzed biomarkers cohort and these results were not part of the main statistical analysis. However, they provided a guidance on the performance of the ELISA assay used.

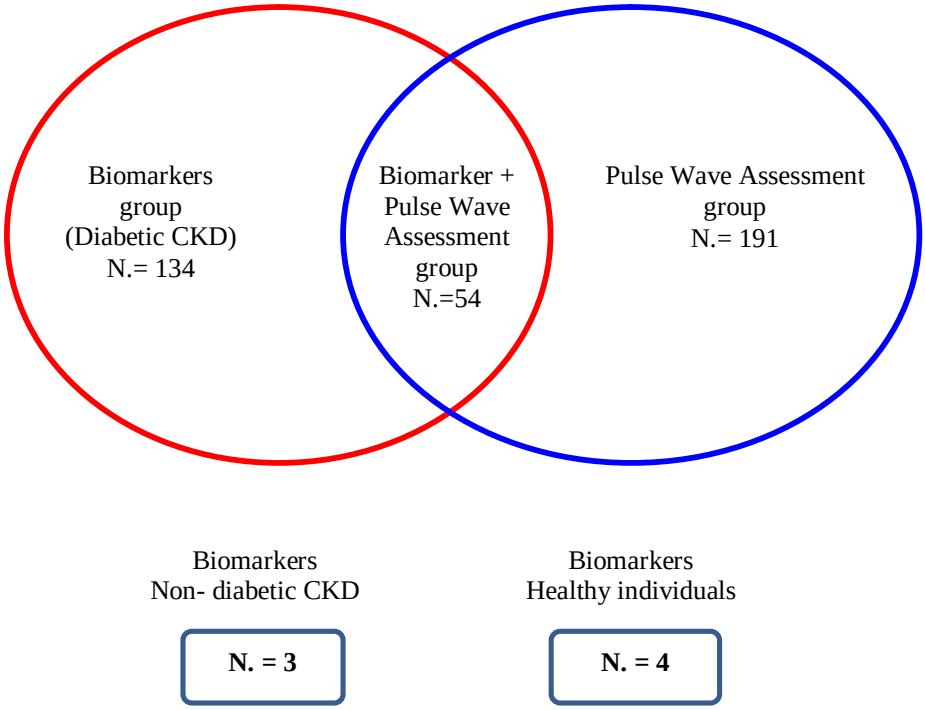


Figure 11: Cohort Study Groups

Discrepancies in patient consents (Pulse Wave Assessment consent vs. Biobanking consent) accounted for the existence of two, partially overlapping cohorts. Those were in part due to the logistic constraints intrinsic to the organization of the outpatient clinics.

1. Clinical History

Clinical history was obtained on the base of the following data:

1. Etiology of CKD, divided in 2 principal groups; DKD and non-diabetic CKD.

2. Age, gender and BMI
3. Social habits:
   a. Smoking: the patients were classified into active smoker (consuming tobacco in regular or sporadic form), Ex-smoker (more than one year without smoking) and non smoker (never smoked).
   b. Alcohol intake: the patients were classified into active consumer (consuming alcohol in regular or sporadic form), Ex-consumer (more than one year without alcohol intake) and non-consumer (never drink alcohol).
4. Presence of CVD:
   a. Cardiac history: angina, myocardial infarction, heart failure, valvuloapthy, cardiac arrhythmias, coronary artery disease, systolic dysfunction, or diastolic dysfunction.
   b. Peripheral vascular disease: Intermittent Claudication
   c. Active pharmacological treatment considering:
      - Antidiabetics: oral hypoglycemic agents or injection therapy (Insulin)
      - Antihypertensives: ACEIs, ARBs, beta-blockers, calcium channels blockers, alpha-blockers, alpha-beta blockers and diuretics.
      - Lipid lowering agents: Statins, Fibrates, Ezetimibe and Omega-3-fatty acids
      - Anti-platelet agents: acetylsalicylic acid, clopidogrel, ticlopidine and triflusal.
      - Iron supplementations and Erythropoiesis Stimulating Agents (ESA) and proton pump inhibitors
      - Calcium and Vitamin D supplementations
      - Potassium chelator (Calcium polystyrene sulfonate; resin calcium) and Phosphorus binders including calcium based (calcium acetate and calcium carbonate) and non calcium based (sevelamer, lanthanum and aluminum).
      - Cinacalcet

2. Blood and urine analysis

Blood and urine were assessed for biochemical parameters at the clinical laboratory of IIS-Fundacion Jimenez Diaz.

1. Plasma PCSK9 and plasma CXCL16 were assessed by ELISA R&D Systems (Minneapolis, USA) at the research laboratory of nephrology.
2. Parameters of kidney functions: serum creatinine and eGFR; calculated by the 4 parameter MDRD formula [333].
3. Parameters of anemia and iron saturation: Hemoglobin (Hb), Iron, total iron binding capacity (TIBC), Ferritin and transferrin.
4. Parameters of bone and mineral metabolism: calcium, phosphorus, magnesium, alkaline phosphatase and lactate dehydrogenase (LDH). ELISA was used to measure intact parathyroid hormone (PTH) (second generation immunoradiometric assay (IRMA) (CA-PTH duo; Scantibodies Laboratory Inc), 25 hydroxyvitamin D {25(OH)D} and 1,25 dihydroxivitamin D {$1,25(OH)_2D$}(radioimmunoassay, DiaSorin).
5. Lipid profile: Serum cholesterol (Total, LDL and HDL) and Triglycerides (TG).
6. Uric acid level.
7. Parameters of inflammation: hsCRP.
8. Parameters of nutritional status: Serum albumin and prealbumin.
9. Parameters of glucose control: HbA1c, random blood glucose and glycosuria.
10. Vasoactive hormones: renin activity (ng/ml/h); [normal level: (orthostatic: 1.3-4) and (supine: 0.2-2.3)] and aldosterone (pg/ml); [normal level: (orthostatic: 40-300) and (supine: 17-130)]. These measures have been recorded in the morning hours (10.00-12.00 h).
11. Ions: sodium and potassium were measured in the same day of the vasoactive hormones. In addition, blood CO_2 was measured by autoanalyzer.
12. Thyroid profile: TSH, Free T3, Free T4.
13. Vitamins: Serum Vitamins A, E, B12 and folic acid levels.
14. Urine examination; including:
    - 24 hours creatinine clearance.
    - Spot urinary creatinine, protein and microalbumin.
    - UACR and UPCR.
    - Spot urinary excretion of sodium and potassium, calcium, phosphorus and magnesium.
    - 24 hours excretion of creatinine, protein, phosphorus and magnesium.

3. Complementary cardiac investigations

1. Basal Electrocardiogram.
2. Transthoracic Echocardiogram to evaluate: All the following data have been specified and provided by the cardiologist.
   - LV diameter,
   - Left atrio-ventricular septum diameter,
   - Ejection fraction,
   - Altered ventricular relaxation pattern: grade I diastolic dysfunction; altered mitral valve flow.
   - LVH: Normal thickness of the LV myocardium is from 6 to 11 mm measured at the very end of diastole. The diagnosis of LVH was made, if the myocardium is more than 11 mm thick [334].
   - Cardiomyopathy (absence, hypertrophic or dilated):
     a. The hypertrophic cardiomyopathy was diagnosed based on the echocardiographic criteria which included; asymmetrical septal hypertrophy, systolic anterior motion of the mitral valve, a small LV cavity, septal immobility, and premature closure of the aortic valve. LV thickness, evaluated at septum and free wall level, is considered abnormal when Ó 15 mm, and defined asymmetrical in presence of a septal to free wall thickness ratio between 1.3 and 1.5 [335].
     b. The dilated cardiomyopathy was identified by the presence of LV dilatation and LV systolic dysfunction with normal LV wall thickness, diagnosis was made with 2-dimensional echocardiography [336, 337].
   - Valvular calcification (absence or presence),
   - Pericardial effusion (absence, mild, moderate or severe)

4. Pulse Wave assessment

The assessment of arterial wave reflection characteristics was performed noninvasively using the SphygmoCor CV Management System (CvMS*)* 2010 software version 9 (AtCor Medical, Sydney, Australia). High-fidelity carotid and femoral arteries pressure waveforms were recorded by applanation ópencil typeô tonometry (SPT-304) of the carotid and femoral pulses. The best high-quality recordings; defined as an in-device quality control (operator index) of over 75%, was used for analysis. The operator index was derived from an algorithm including average pulse height variation, diastolic variation (<5%), and the maximum rate of rise of the peripheral waveform.

A brachial BP measurement was taken using an automatic sphygmomanometer. The patient was sitting or lying comfortably and allowed to rest approximately 5 minutes prior to taking the brachial BP measurement to ensure stable hemodynamics. At least 2 minutes elapsed between taking the brachial BP and recording a pressure waveform using the tonometer. Then, diastolic and systolic BP, height, weight and BMI were entered in the corresponding fields on the Study Screen. In general, pressure waveforms were gated with simultaneous electrocardiographs recording.

Aortic (Carotid-Femoral) Pulse Wave Velocity

The standard method of measuring PWV is to record a proximal (located toward the centre of the body) and a distal (located far from the centre of the body) pressure waves at two different sites on the arterial tree. The Aortic PWV is recorded from simultaneous measurement of the pressure wave propagation from the carotid artery (proximal) to the femoral artery (distal). Foot-to-foot PWV was calculated by determining the delay between the appearance of the pressure waveform foot in the carotid and femoral sites (qt; transit time). The measurement of the tonometry transit distance (TTD) was made using a measuring tape on the surface of the body connecting the carotid measuring site with the femoral measuring site then multiplying the value by a factor of (0.8) [338]. Then the device software automatically estimated the CF-PWV (m/sec). The results displayed compared to age-based reference range (Figure 12).

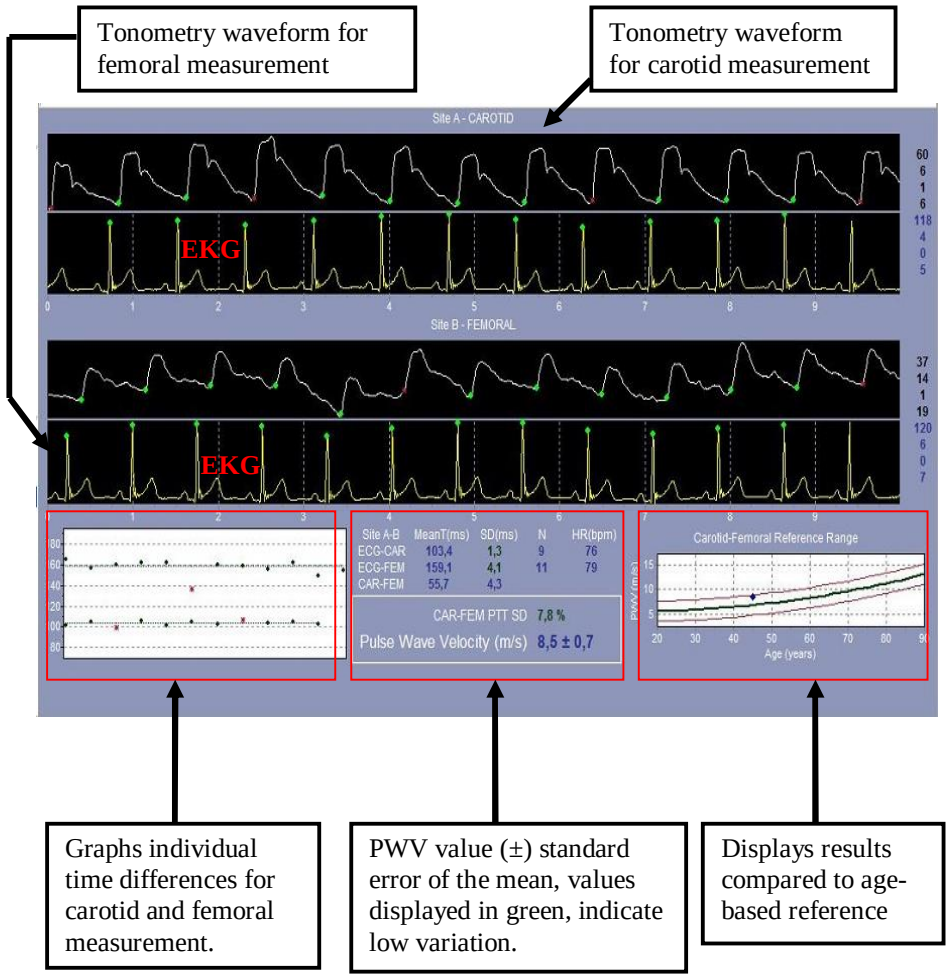


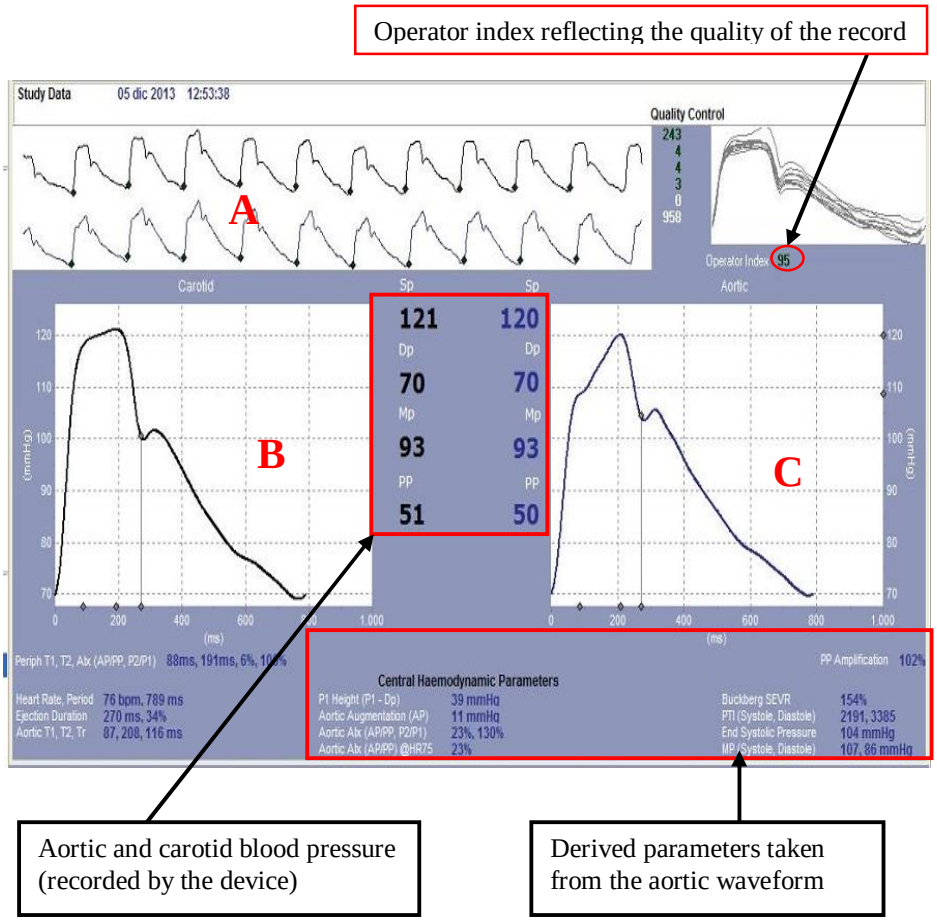
Figure 12: CF-PWV record data of one of our patient as obtained by SphygmoCor device

The Delta PWV was calculated as follows= (measured PWV) - (Upper limit of the age-adjusted PWV values for the general population). The PWV values for the general population have been obtained from data of 16 867 subjects and patients from 13 different centers across eight European countries [279]. All patients with positive values were considered to have an abnormally high PWV for their age, while those Within the normal limits were assigned a value of zero. All patients were included in the delta PWV statistical analysis.

Aortic Pulse wave analysis (PWA)

The aortic pressure waveform was derived non-invasively from the carotid femoral pulse wave using applanation tonometry. Central pressure values were estimated from carotid measurements using the software's mathematical transfer function. The following PWA parameters related to the amplification and temporal characteristics of the reflecting wave were used as independent variables in the present study (Figure 13):

- Aortic Systolic pressure (mmHg)
- Aortic Pulse Pressure (mmHg), calculated by the difference between aortic systolic and diastolic BP; [Aortic pulse pressure = Aortic systolic BP ï Aortic diastolic BP]
- Aortic Augmentation Pressure (mmHg); the pressure wave created by left ventricular contraction propagates forward until meeting sites of resistance that reflect the wave backward; thus, stiffer artery walls result in earlier wave reflection. When the reflected wave returns during systole rather than diastole, systolic pressure is increased or ñaugmented.ò Augmentation Pressure is calculated by the difference between the reflected wave (P2) and forward wave (P1) (Augmentation Pressure=P2-P1) is a measure of how much the early reflected wave contributes to central systolic pressure.



A 9 seconds of captured carotid waveform and corresponding derived aortic waveform.
B average of the captured carotid waveforms
C average of the derived aortic waveforms

Figure 13: Aortic PWA record data of one of our patients as obtained by the SphygmoCor device

- Aortic Augmentation Index (AIX) represents the level of augmentation measured and is expressed as a percentage of the pulse pressure (Aortic AIX = Aortic Augmentation Pressure / Aortic Pulse Pressure).
- Normalized Aortic Augmentation Index AIX@75; expressed as a percentage (%) defined as AIX normalized to a fixed heart rate of 75 beats per minute.
- Subendocardial viability ratio (SEVR) or Buckberg Index; is a tonometric noninvasive measure of myocardial perfusion (as coronary artery perfusion takes place primarily during diastole) relative to cardiac workload (myocardial contraction). SEVR is measured as the ratio of diastolic pressure time index (DPTI) to tension time index (TTI); [DPRI/TTI] and is expressed as a percentage (%).

5. Statistical Methodology

Data analyses were performed using statistical software R version 3.0.1. Quantitative variables were described by mean and standard deviation or by median and inter-quartile ranges (IQR); 25% percentile and 75% percentile. Qualitative variables were described by frequency tables and contingency tables. Associations between quantitative and qualitative variables were assessed using Studentôs t test or Mann-Whitney test when comparing two groups, and ANOVA or Kruskal-Wallis test when comparing three or more groups. Correlations between quantitative variables were evaluated using Spearman correlation coefficient. Scatter plots and box plots were used to describe associations between variables. In order to identify potential predictors of quantitative outcomes, multivariable linear regression models were fitted. Models were built using forward stepwise procedures in order to maximize R-Squared with the smallest

number of predictor variables. The statistical significance of variables in the models was assessed by ANOVA test.

The significance level was set as the following:

- P value <0.01 = highly Significant
- P value <0.05 = Significant
- P value >0.05 and <0.1= trend towards association

Univariate and multivariable analyses were performed with plasma PCSK9, plasma CXCL16, cfPWV, Aortic AIX@75, and SEVR as dependent variables. In order not to overload the results section, only statistically significant results are presented.

RESULTS

We performed a cross-sectional analysis of baseline data from two partially overlapping prospective cohorts (Figure 11).

One cohort, that we will term the ñBiomarkerò cohort, included patients with diabetic CKD not on dialysis on regular follow up visits in the diabetic nephropathy clinic at Fundación Jiménez Díaz University Hospital. In this cohort of diabetic CKD patients (n.=134) biomarkers (plasma PCSK9 and CXCL16) were assessed.

A second cohort, that we will term the ñPulse Wave Assessmentò cohort, included 191 individuals. Of these, 153 were CKD patients and 38 non-CKD patients with hypertension or high cardiovascular risk.

Both cohorts had in common 54 diabetic CKD patients, for whom both pulse wave assessment and biomarkers data were available. These shared patients were analyzed together with the rest of patients from their respective cohorts.

1. Biomarkers cohort

The biomarker cohort consisted of 134 subjects; all of them were diabetic and had CKD. Most patients were in CKD stage 3 (49/134, 37%). Mean±SD (standard deviation) of age was 67.9±13.9 years and most of the patients were males (92/134, 69%). This population was borderline obese with mean BMI 29.7±4.2 kg/m^2.

Main patient characteristics (Table 5) and pulse wave characteristics (Table 6) were summarized below.

Table 5: Main characteristics of patients in the biomarkers cohort (n=134)

Variable		Value
Total patients n (%)		134 (100)
DM n (%)		134 (100)
Hypertension n (%)		128 (95.5)
CVD n (%)		35 (26)
CKD Etiology n (%)	Non CKD (control)	0
	DM	122 (91)
	Vascular	12 (9)
	Glomerulonephritis	0
	Unknown	0
CKD Stages n (%)	Non CKD (control)	0 (0)
	Stage 1	11 (8.2)
	Stage 2	40 (29.9)
	Stage 3A	14 (10.4)
	Stage 3B	35 (26.1)
	Stage 4	34 (25.4)
	Stage 5	0
Cigarette smoking n (%)	Non smoker	62 (46.3)
	Active smoker	25 (18.7)
	Ex-smoker	47 (35.1)
Alcohol consumption n (%)	Non consumer	82 (61.2)
	Active consumer	41 (30.6)
	Ex-consumer	11 (8.2)
Age (years)		67.9±13.9
Gender n (%)	Males	92 (68.7)
	Females	42 (31.3)
Height (cm)		165.1±10.3
Weight (kg)		81.0±14.9
Body Mass Index (kg/m^2)		29.7±4.2

Table 6: Pulse Wave characteristics of patients in the biomarkers cohort (n=134)

Variable		Value
Total patients n (%)		134 (100)
Systolic Blood Pressure (mmHg)		143.1±17.8
Diastolic Blood Pressure (mmHg)		75.2±11.8
Mean Blood Pressure (mmHg)		100.1±12.4
Pulse Pressure (mmHg)		67.9±17.1
Mean PWV (m/sec)		11.8±3.3
SD PWV (m/sec)		1.3±0.7
CF-PTT (SD) (%)		10.5±4.4
PWV Reference Range n (%)	within normal	26 (48.1)
	high normal	5 (9.3)
	above normal	23 (42.6)
Normal values for age (m/sec)		12.5±2.4
Delta PWV (m/sec)		1.1±2.0
Aortic Systolic pressure (mmHg)		140.7±18.4
Aortic Pulse Pressure(mmHg)		63.0±16.6
Aortic AIx@75 (%)		24.1±14.1
SEVR; Buckberg Index (%)		143.5±27

(CF-PTT: Carotid Femoral Pulse Transit Time)

Mean eGFR was 56.3±23.0 ml/min/1.73m^2 and the median (IQR) UACR was 123.6 (27.1, 386.7). Regarding the UACR, 35 patients (26%), 56 patients (42%), 26 patients (19%) and 17 patients (13%) had UACR of < 30, 30 to 299, 300 to 1000 and > 1000 mg /g respectively.

The baseline urinalysis parameters (Table 7), distribution of UACR (Table 8), serum analysis parameters (Table 9) and echocardiograms (Table 10) .

Table 7: Urinalysis parameters of the biomarkers cohort (134 diabetic CKD patients)

Urinalysis parameters / (n. of patients)	Value
Total patients	134
Creatinine Clearance (ml/min) / (136)	66.9±34.1
Diuresis (ml/24h) / (136)	1892.4±554.3
Total Proteinuria (mg/24h) / (134)	320.0 (135.0, 683.5)
Glycosuria (mg/dl) / (136)	38.4±58.1
Spot Proteinuria (mg/dl) / (136)	17.7 (7.6, 38.0)
UPCR (mg/g) / (136)	261.2 (127.9, 698.5)
Total Microalbuminuria (mg/24h) / (78)	207.4 (36.5, 518.6)
UACR (mg/g) / (137)	123.6 (27.1, 386.7)
Spot Creatinuria (mg/dl) / (137)	69.0±42.1
Urinary Sodium (mmol/L) / (135)	86.0±35.3
Urinary Potassium (mmol/L) / (135)	34.3±15.0
Urinary Magnesium (mg/dl) / (135)	3.9±2.1
Urinary Magnesium (mg/24h) / (135)	71.3±35.7
Urinary Magnesium (mg/g Cr.) / (135)	0.063± 0.030
FEMg (%) / (135)	6.6 ±4.3
Urinary Calcium (mg/dl) / (135)	5.1±7.2
Phosphaturia (mg/dl) / (135)	34.2±14.1
Phosphaturia (mg/24h) / (135)	620.9±257.2
Phosphaturia (mg/mg Cr.) / (135)	0.546±0.200

Table 8: Distribution of UACR in the biomarkers cohort (134 diabetic CKD patients)

UACR (mg/g)	Number of patients (%)
<30	35 (26.1)
30-299	56 (41.8)
300-1000	26 (19.4)
>1000	17 (12.7)
Total	134 (100)

Table 9: Serum analysis parameters of the biomarkers cohort (134 diabetic CKD patients)

Serum analysis parameters / (Number of patients)	Biomarkers cohort
Total patients	134
Serum Creatinine (mg/dl) / (137)	1.4±0.6
GFR (MDRD) (ml/min/1.73 m^2) / (137)	56.3±23.0
Serum Glucose (mg/dl) / (137)	141.9±50.2
Serum Uric Acid (mg/dl) / (137)	6.5±1.7
Serum HbA1C (%) / (137)	7.5±1.2
Haemoglobin (g/dl) / (137)	13.6±1.7
Serum Albumin (g/dl) / (137)	4.1±0.4
Serum hsCRP (mg/dl) / (65)	1.2±3.3
Serum Prealbumin (mg/dl) / (116)	27.3±6.7
Serum Transferrin (mg/dl) / (137)	251.9±52.3
Serum Ferritin (ng/ml) / (137)	84.5 (45.3, 176.3)
Serum Total Cholesterol (mg/dl) / (137)	154.4±32.6
Serum LDL Cholesterol (mg/dl) / (137)	82.8±26.1
Serum HDL Cholesterol (mg/dl) / (137)	44.8±13.8
Serum Triglycerides (mg/dl) / (137)	136.5±84.0
Plasma Renin (ng/ml/hr) / (115)	6.8 (1.8, 28.0)
Plasma Aldosterone (pg/ml) / (123)	97.7 (70.9, 144.0)
Serum CO_2 (mEq/L) / (130)	27.4±3.6
Serum LDH (IU/l) / (137)	398.3±102.5
Serum Sodium (mmol/l) / (137)	139.8±2.8
Serum Potassium (mmol/l) / (137)	4.6±0.5
Serum Magnesium (mg/dl) / (135)	1.9±0.3
Serum Calcium (mg/dl) / (137)	9.5±0.5
Serum Phosphorus (mg/dl) / (137)	3.5±0.6
Serum Alkaline Phosphatase (IU/l) / (137)	84.0±32.6
Serum Iron (εg/dl) / (137)	73.8±25.1
Serum TIBC (εg/dl) / (137)	320.6±67.6
Serum intact PTH (pg/ml) / (134)	52.7 (35.0, 93.6)
Serum 25(OH)D (ng/ml) / (134)	20.1±10.3
Serum $1,25(OH)_2D$ (pg/ml) / (89)	30.1±12.3
Serum Vitamin B12 (pg/ml) / (124)	427.7±204.6
Serum Folic Acid (ng/ml) / (123)	7.8±3.7
Serum TSH (εIU/ml) / (127)	2.2±1.2
Serum Free T3 (pg/ml) / (121)	3.1±0.6
Serum Free T4 (ng/dl) / (128)	1.2±0.3
Serum Vitamin A (mg/l) / (107)	0.7±0.3
Serum Vitamin E (εg/ml) / (107)	14.5±5.0
Plasma PCSK9 (ng/ml) / (134)	309.8±113.9
Plasma CXCL16 (ng/ml) / (134)	4.0±0.9

Table 10: Echocardiograms for biomarkers cohorts

Variable		Value
Total Patients n (%)		113 (100)
Ejection Fraction (%)		58.9±7.8
Left Ventricular Diameter (mm)		43.4±5.8
Interventricular septum (mm)		10.2±1.4
Left Ventricular Hypertrophy n (%)		48 (42.5)
Altered Relaxation n (%)		80 (70.8)
Cardiomyopathy n (%)	None	100 (88.5)
	Hypertrophic	11 (9.7)
	Dilated	2 (1.8)
Valvular Calcification n (%)		19 (16.8)
Pericardial Effusion n (%)		2 (1.8)

The majority of the patients were receiving antidiabetic; oral hypoglycemic agents (84/134, 63%) and Insulin (90/134, 67%) or antihypertensive (134/134, 100%) medications. As well as 113/134 (84%) were on lipid lowering therapy. Over 67% of the patients were taking anti-platelet therapy. A minority of patients were receiving vitamin D supplementation (48/134, 36%), phosphate binders (12/134, 9%), or ESAs (7/134, 5%). Patientsô medications (Table 11) and dosage for selected medications (Table 12) were summarized below.

Additionally to the study of the diabetic CKD cohort, biomarkers were assessed in 4 healthy subjects and 3 non-diabetic CKD patients. This was for the purpose of assessing the performance of the ELISA assay (technique control) and these biomarker results were not used for the main statistical analysis.

For the healthy subjects; mean age was 36.8±8.9 years, most of them were males (3, 75%), mean plasma PCSK9 was 261±76 ng/ml and mean plasma CXCL16 was 3.1±0.3 ng/ml (Annex I).

For the non-diabetic CKD patients; mean age was 71.0±8.5 years, most of them were males (2, 66.7%), mean plasma PCSK9 was 341±106 ng/ml and mean plasma CXCL16 was 5.3±0.6 ng/ml. Mean eGFR was 25.0±11.8 and mean UACR was 704.4±421.6 (Annex I). Further characteristics for the non-diabetic CKD control patients, laboratory parameters, medications and echocardiograms are available in Annex II.

Table 11: Medications of the patients in the biomarkers cohorts.

Medication	Number of patients (%)
Total patients	134 (100)
Any vitamin D or VDR activator	48 (35.8)
Paricalcitol (mcg/w)	16 (11.9)
Calcifediol (IU/w)	42 (31.3
Calcitriol (mcg/w)	4 (3)
Cinacalcet (mg/w)	1 (0.7)
Any phosphate binder	12 (9)
Lanthanum	1 (0.7)
Sevelamer	4 (3)
Aluminium- based phosphate binders	6 (4.5)
Calcium-based phosphate binders	3 (2.2)
Calcium Supplement	5 (3.7)
Calcium Polystyrene Sulfonate (Potassium chelator)	10 (7.5)
Iron supplement	33 (24.6)
ESAs	7 (5.2)
Oral hypoglycemic agents	84 (62.7)
Insulin	90 (67.2)
Any lipid lowering agent	113 (84.3)
Statin	110 (82.1)
Fibrate	18 (13.4)
Ezetimibe	14 (10.4)
Omega 3-Fatty Acids	14 (10.4)
Any anti-hypertensive *	134 (100)
Any RAAS blocker	131 (97.8)
ACEIs	60 (44.8)
ARBs	95 (70.9)
Spironolactone	4 (3)
Calcium Channel Blockers	79 (59)
Beta Blockers	37 (27.6)
Alpha Blockers	30 (22.4)
Alpha & Beta Blockers	1 (0.7)
Diuretics	94 (70.1)
Proton Pump Inhibitors	54 (40.3)
Anti-platelets	90 (67.2)

* Including RAAS blockers, calcium channel blockers, beta blockers, alpha & beta blockers and diuretics

Table 12: Dosage for selected medications; only patients received the medications were analyzed.

Medication	Mean±SD or Median (IQR)
Paricalcitol (mcg/w)	3.0±2.2
Calcifediol (UI/w)	2160 (1440, 2880)
Calcitriol (mcg/w)	0.75 (0.70, 18.00)

Plasma PCSK9

In the cohort of diabetic CKD patients, the mean±SD of the plasma PCSK9 level were 309.8 ± 113.9 ng/ml. We explored the association of plasma PCSK9 levels with clinical, echocardiogram, therapeutic and laboratory parameters. Plasma PCSK9 values according to CKD stages (Figure 14), UACR (Figure 15) and lipid lowering therapy (Figure 16) are shown below.

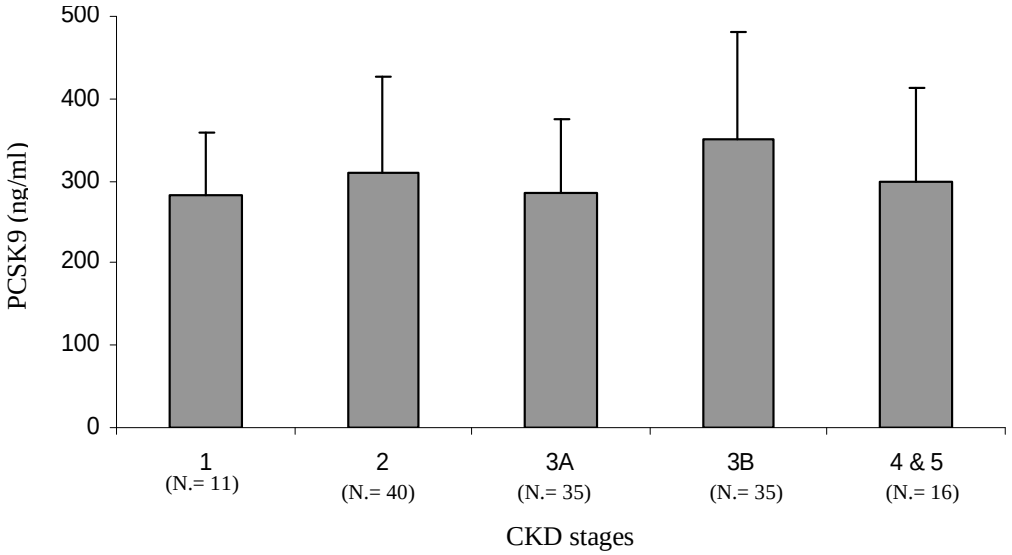


Figure 14: Plasma PCSK9 values according to CKD stages

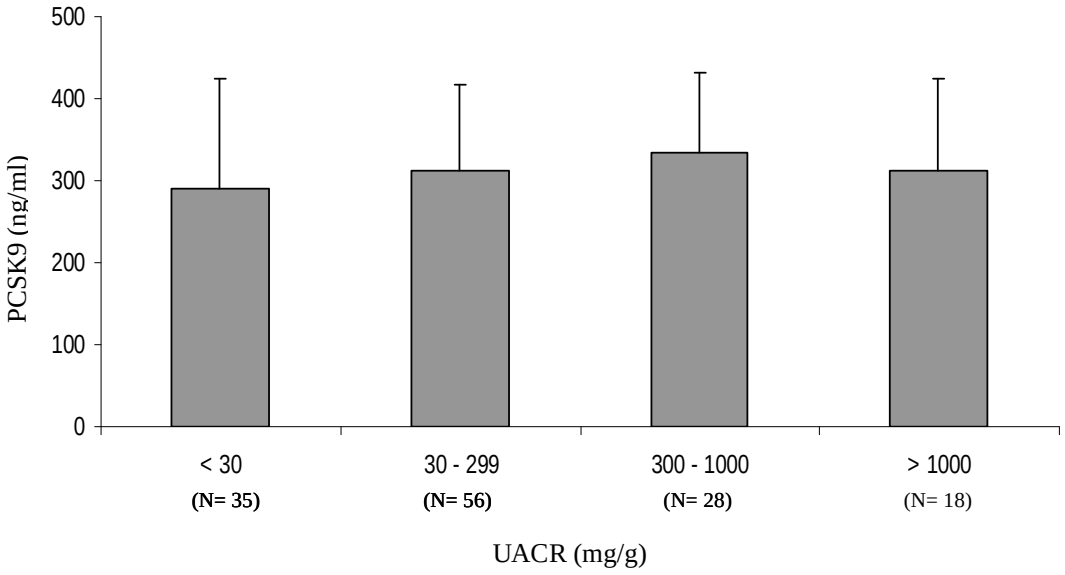


Figure 15: Plasma PCSK9 values according to UACR

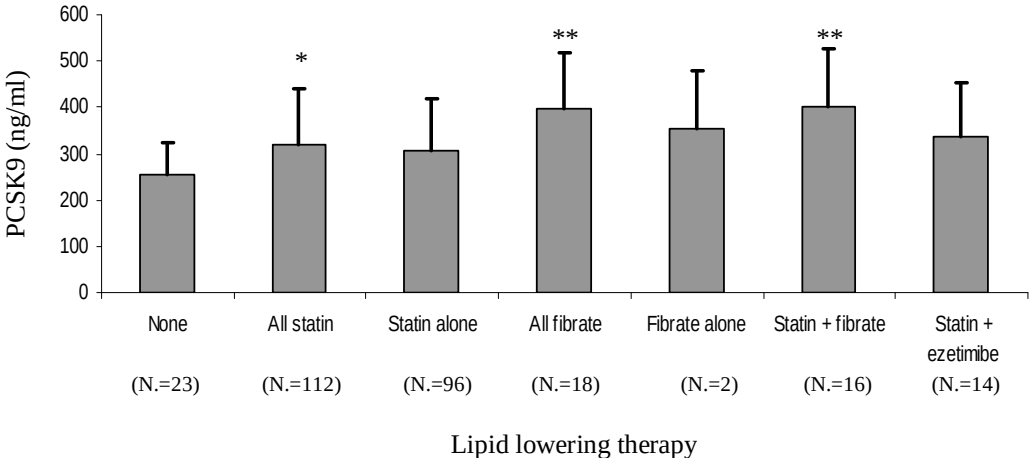


Figure 16: Plasma PCSK9 values according to lipid lowering therapy categories. * $p < 0.05$, ** $p < 0.01$ vs none

The univariate analysis of plasma PCSK9 (ng/ml) with other qualitative variables (Table 13) and quantitative variables (Table 14), showed that PCSK9 had a significant positive correlation with lipid lowering therapy (statin, fibrate or both together), serum TIBC, vitamin E, plasma renin and phosphaturia (Figure 17). Also there was a trend towards a positive correlation with total serum cholesterol.

Table 13: Correlations between plasma PCSK9 (ng/ml) and qualitative variables in univariate analysis. Only statistically significant ($p < 0.05$) results or trends ($p \geq 0.05$ to $p < 0.1$) are shown.

Variable	N	Mean	SD	P value
Statin				
No	24	264.62	73.3	
Yes	110	319.62	118.92	0.0050
Fibrate				
No	116	296.16	106.69	
Yes	18	397.28	122.79	0.0004
Statin + Fibrate				
No	118	297.17	106.68	
Yes	16	402.69	125.5	0.0004

Table 14: Correlations between plasma PCSK9 (ng/ml) and quantitative variables in univariate analysis. Only statistically significant ($p < 0.05$) results or trends ($p \geq 0.05$ to $p < 0.1$)] are shown.

Variable	N	Coefficient	P value
Plasma Renin (ng/ml/hr)	113	0.1986	0.0350
Phosphaturia (mg/mg Cr.)	132	0.2565	0.0030
Serum TIBC (µg/dl)	134	0.2087	0.0155
Serum Vitamin-E (µg/ml)	105	0.2132	0.0290
Serum Total Cholesterol (mg/dl)	134	0.1566	0.0708

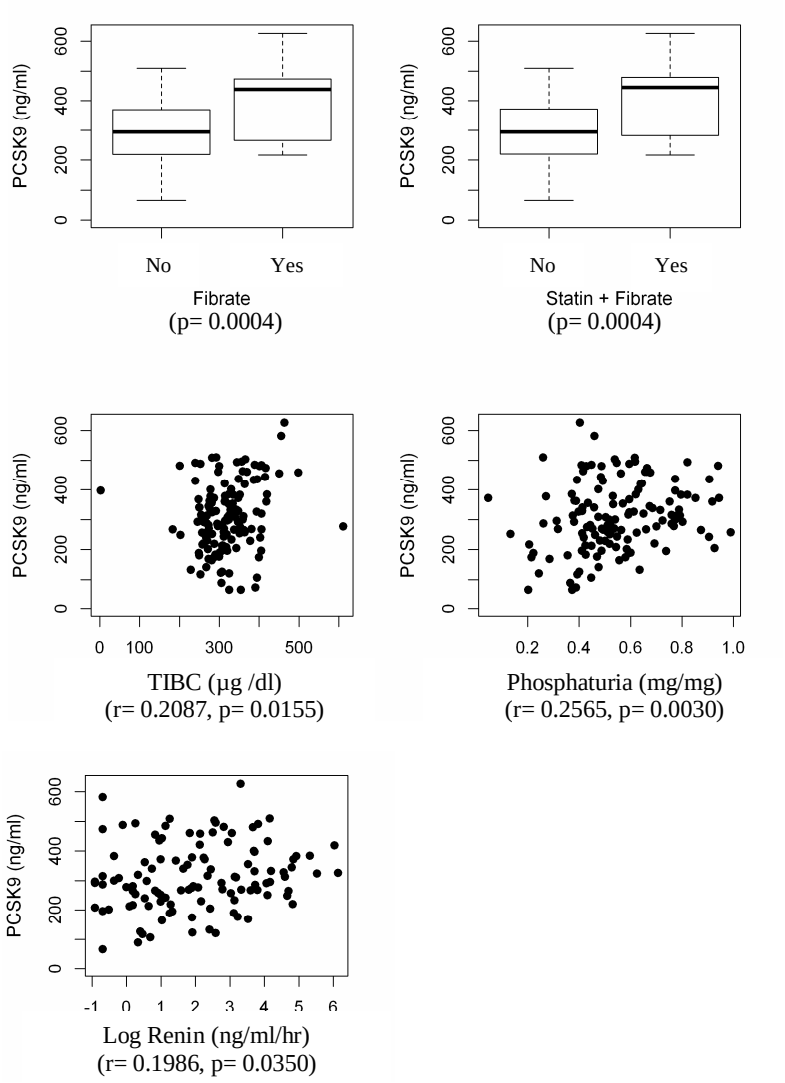


Figure 17: Statistically significant correlations with PCSK9

In multivariate models, the only parameters that remained independently significantly positively correlated with plasma PCSK9 were therapy with fibrate alone or fibrate with statin, serum TIBC, Log Renin and phosphaturia. In general, the multivariate models (Table 15) explain very little of the PCSK9 variability. The best R squared obtained was 0.20.

Table 15: Different multivariate models for predictors of PCSK9 (ng/ml) levels

Model A

	Coefficient	95% CI		p value
Intercept	-16.41	-224.9	192.1	0.8760
Statin	31.99	-23.54	87.53	0.2553
Serum Total Cholesterol (mg/dl)	0.638	-0.154	1.43	0.1130
Phosphaturia (mg/mg Cr)	85.83	-38.56	210.2	0.1737
Serum TIBC (µg/dl)	0.521	0.185	0.858	0.0028
Log Renin (ng/ml/h)	8.529	-3.557	20.62	0.1642
Log Vitamin-E (µg/ml)	-10.25	-99.15	78.64	0.8192

R squared = 0.1551

Model B

	Coefficient	95% CI		p value
Intercept	123.717	-102.984	350.419	0.2810
Statin + Fibrate	99.25	25.049 1	73.451	0.0093
Serum Total Cholesterol (mg/dl)	0.519	-0.252	1.29	0.1845
Phosphaturia (mg/mg Cr)	94.581	-26.109	215.27	0.1229
Serum TIBC (µg/dl)	0.339	-0.017	0.694	0.0616
Log Renin (ng/ml/h)	12.571	0.692	24.45	0.0383
Log Vitamin-E (µg/ml)	-33.944	-122.191	54.302	0.4466

R squared = 0.2074

Plasma CXCL16

In the cohort of diabetic CKD patients, the mean±SD of the plasma CXCL16 levels were 4.0±0.9 ng/ml. We explored the association of plasma CXCL16 levels with clinical, echocardiogram, therapeutic and laboratory parameters. Plasma CXCL16 values according to CKD stages (Figure 18) and UACR (Figure 19) are shown below.

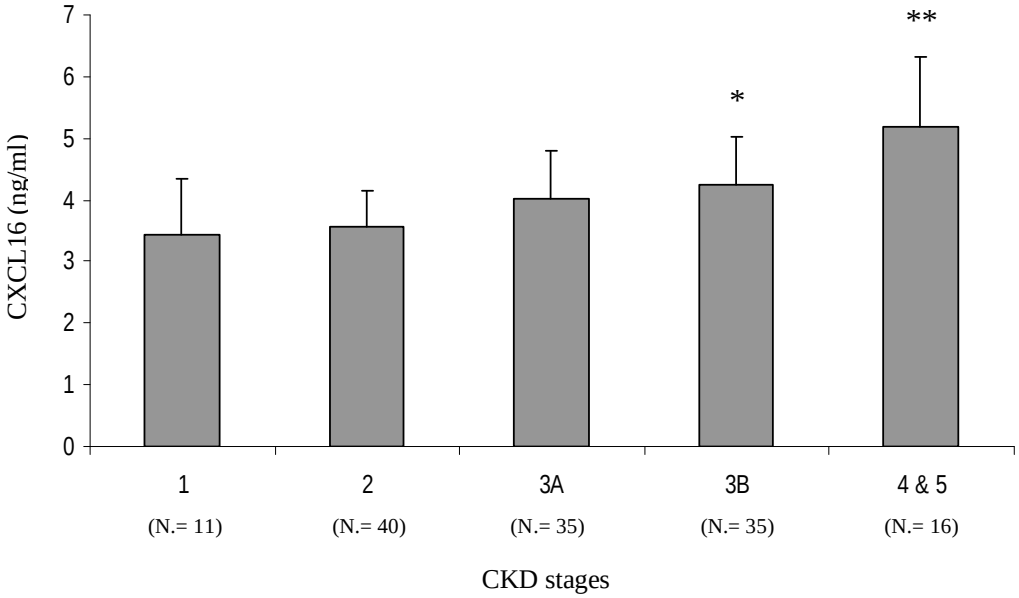


Figure 18: Plasma CXCL16 values according to CKD stages. * $p < 0.05$; ** $p < 0.01$ vs stage 1

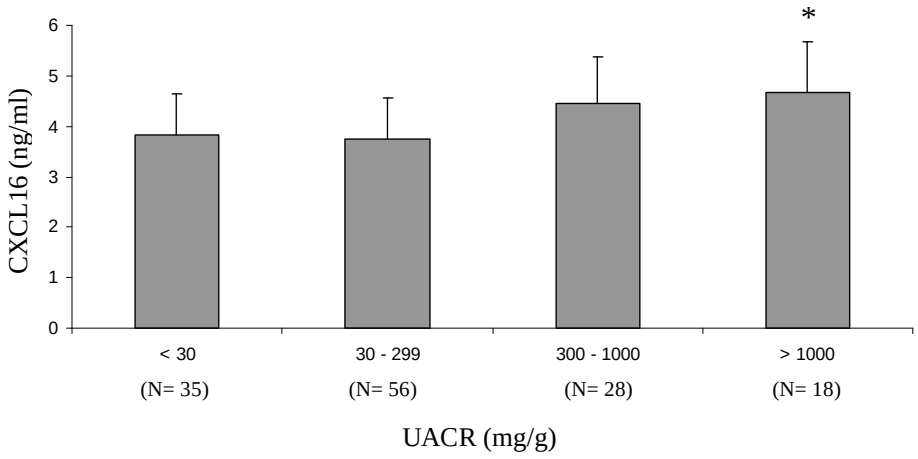


Figure 19: Plasma CXCL16 values according to UACR. * $p < 0.01$ vs UACR< 30 mg/g

The univariate analysis of plasma CXCL16 (ng/ml) with other qualitative variables (Table 16) and quantitative variables (Table 17), showed a significant positive correlation between CXCL16 and history of CVD or treatment with paricalcitol, calcifediol, sevelamer, aluminum containing phosphate binders, potassium chelation therapy with calcium polystyrene sulfonate (resin calcium), iron supplementation, ESAs, oral hypoglycemic agents and beta blockers.

Table 16: Correlations between plasma CXCL16 (ng/ml) and qualitative variables in univariate analysis. Only statistically significant ($p < 0.05$) results or trends ($p \geq 0.05$ to $p < 0.1$)] are shown.

Variable	N	Mean	SD	P value
DM	134	4.001	0.927	0.0200
CVD				
No	99	3.88	0.86	
Yes	35	4.33	1.03	0.0132
Paricalcitol				
No	118	3.91	0.85	
Yes	16	4.65	1.19	0.0024
Calcifediol				
No	92	3.84	0.83	
Yes	42	4.35	1.03	0.0027
Sevelamer				
No	130	3.950	0.874	
Yes	4	5.752	0.584	0.0000
Aluminum-Based Phosphate binders				
No	128	3.978	0.916	
Yes	6	5.150	0.760	0.0025
Calcium polystyrene sulfonate				
No	124	3.966	0.940	
Yes	10	4.683	0.638	0.0110
Iron supplement				
No	101	3.894	0.769	
Yes	33	4.423	1.245	0.0225
ESAs				
No	127	3.954	0.898	
Yes	7	5.244	0.763	0.0001
Oral hypoglycemic agents				
No	50	4.208	0.901	
1 drug	46	4.154	0.976	
2 drugs	29	3.706	0.895	
3 drugs	9	3.380	0.595	0.0135
Beta Blockers				
No	97	3.929	0.861	
Yes	37	4.291	1.084	0.0427
Ezetimibe				
No	120	3.979	0.933	
Yes	14	4.473	0.898	0.0616

Table 17: Correlations between plasma CXCL16 (ng/ml) and quantitative variables in univariate analysis. Only statistically significant ($p < 0.05$) results or trends ($p \geq 0.05$ to $p < 0.1$)] are shown.

Variable	N.	Coefficient	P value
Age (years)	134	0.1942	0.0246
Diastolic BP (mmHg)	134	- 0.2089	0.0154
Pulse Pressure (mmHg)	134	0.1865	0.0310
Paricalcitol dosage (mcg/w)	134	0.2221	0.0099
Calcifediol dosage (IU/w)	134	0.2222	0.0099
GFR (MDRD) (ml/min/1.73 m^2)	134	- 0.5116	0.0000
Total Proteinuria (mg/24h)	134	0.3226	0.0001
UACR (mg/g)	134	0.3024	0.0004
Hemoglobin (g/dl)	134	- .03017	0.0004
Serum Albumin (g/dl)	134	- 0.3520	0.0000
Serum CO_2 (mEq/l)	127	- 0.1911	0.0314
FEMg (%)	132	0.3256	0.0001
Serum Calcium (mg/dl)	134	- 0.2284	0.0079
Serum Phosphorus (mg/dl)	134	0.2230	0.0096
Serum Alkaline phosphatase (IU/l)	134	0.2929	0.0006
Serum TIBC (µg/dl)	134	- 0.2399	0.0052
Serum intact PTH (pg/ml)	131	0.3612	0.0000
Serum 1,25 $(OH)_2D$ (pg/ml)	87	- 0.2172	0.0433
Serum Folic acid (ng/ml)	121	- 0.1992	0.0285
Interventricular septum (mm)	113	0.1748	0.0641
SEVR; Buckberg index (%)	54	- 0.2487	0.0697

In addition, positive correlations were found between CXCL16 and age, pulse pressure, 24-hour urinary protein, UACR, fractional excretion of magnesium (FEMg), serum phosphorus, alkaline phosphatase and intact PTH. CXCL16 had a negative correlation with diastolic BP, eGFR (MDRD), Hb, serum TIBC, serum albumin, CO_2, serum calcium, 1,25$(OH)_2D$ and folic acid (Figure 20).

There was also a trend towards a positive correlation between CXCL16 and ezetimibe intake and interventricular septum thickness and a trend towards a negative correlation with SEVR.

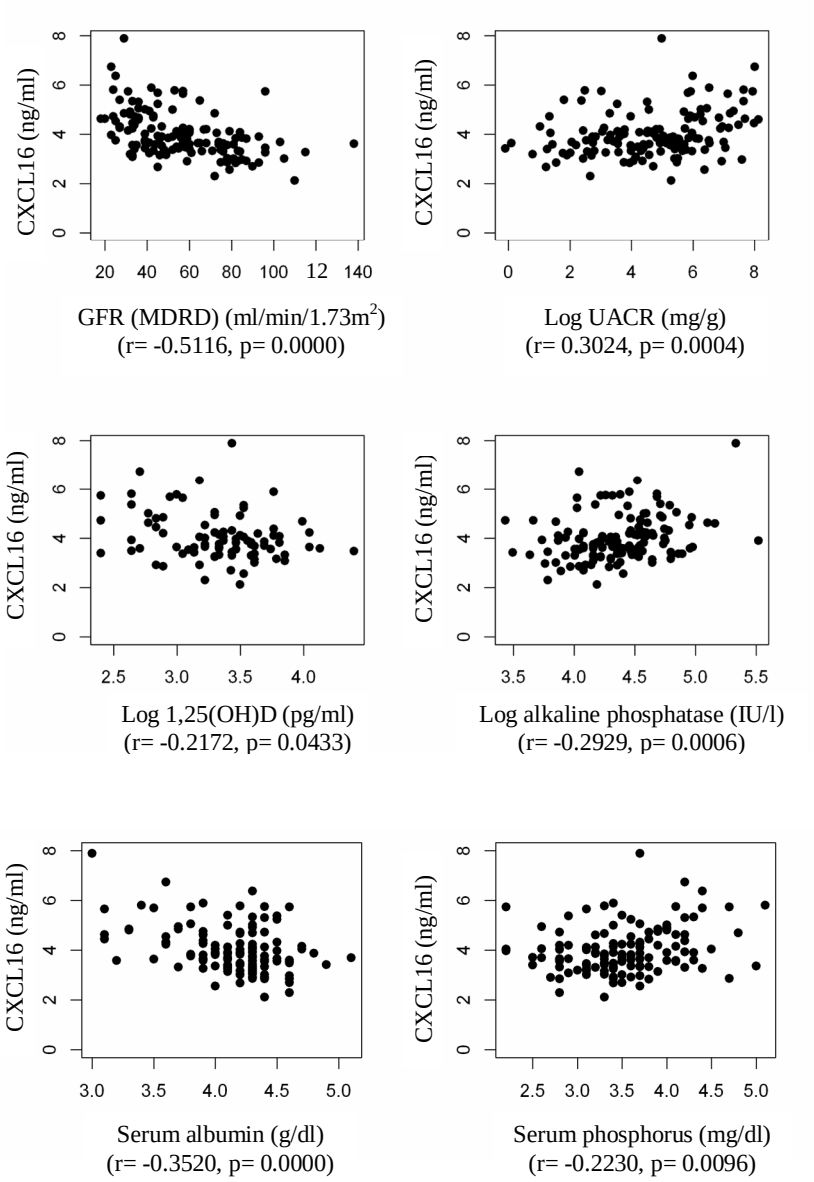


Figure 20: Statistically significant correlations with CXCL16

Several multivariate models (Table 18) showed that GFR and serum albumin remained independently significantly negatively correlated with plasma CXCL16, while UACR and serum alkaline phosphatase were independently positively correlated with plasma CXCL16. In addition, there was a trend towards a negative correlation with Log $1,25(OH)_2D$ and a trend towards a positive correlation with serum phosphorus. The best R squared obtained was 0.34.

Table 18: Different multivariate models for predictors of CXCL16 (ng/ml) levels

Model A

	Coefficient	95% CI		p value
Intercept	3.302	0.578	6.026	0.0181
GFR (MDRD) (ml/min/1.73 m^2)	-0.019	-0.027	-0.010	0.0000
Log UACR (mg/g)	0.098	-0.012	0.208	0.0792
Serum Phosphorus (mg/dl)	0.181	-0.130	0.492	0.2501
Log Alkaline phosphatase (IU/l)	0.380	-0.164	0.924	0.1684
Log $1,25\ (OH)_2D$ (pg/ml)	-0.311	-0.755	0.132	0.1661

R squared = 0.347

Model B

	Coefficient	95% CI		p value
Intercept	6.289	1.867	10.711	0.0059
Age (years)	-0.007	-0.022	0.008	0.3472
GFR (MDRD) (ml/min/1.73 m^2)	-0.023	-0.034	-0.013	0.0000
FEMg (%)	0.001	-0.048	0.050	0.9621
Serum Calcium (mg/dl)	-0.147	-0.523	0.229	0.4384
Serum Phosphorus (mg/dl)	0.240	-0.089	0.570	0.1499
Serum Alkaline phosphatase (IU/l)	0.005	0.000	0.014	0.0393
Serum intact PTH (pg/ml)	-0.002	-0.004	0.001	0.1309
Serum 1,25 $(OH)_2D$ (pg/ml)	-0.014	-0.029	0.001	0.0691

R squared = 0.3426

Model C

	Coefficient	95% CI		p value
Intercept	6.282	1.778	10.787	0.0069
Age (years)	-0.008	-0.023	0.008	0.3336
GFR (MDRD) (ml/min/1.73 m^2)	-0.024	-0.034	-0.013	0.0000
Calcifediol dosage (IU/w)	0.000	-0.000	0.000	0.7468
Paricalcitol dosage (mcg/w)	-0.015	-0.135	0.104	0.7985
FEMg (%)	0.002	-0.048	0.053	0.9263
Serum Calcium (mg/dl)	-0.141	-0.525	0.243	0.4662
Serum Phosphorus (mg/dl)	0.239	-0.095	0.572	0.1579
Serum Alkaline phosphatase (IU/l)	0.007	-0.000	0.014	0.0505
Serum intact PTH (pg/ml)	-0.002	-0.004	0.001	0.1342
Serum 1,25 $(OH)_2D$ (pg/ml)	-0.014	-0.030	0.001	0.0666

R squared = 0.3267

Model D

	Coefficient	95% CI		p value
Intercept	8.000	6.505	9.495	0.0000
GFR (MDRD) (ml/min/1.73 m^2)	-0.016	-0.022	-0.010	0.0000
Serum Albumin (g/dl)	-0.751	-1.126	-0.376	0.0001

R squared = 0.3002

Model E

	Coefficient	95% CI		p value
Intercept	8.075	5.727	10.424	0.0000
Age (years)	-0.008	-0.019	0.003	0.1498
GFR (MDRD) (ml/min/1.73 m^2)	-0.018	-0.025	-0.011	0.0000
Log UACR (mg/g)	0.048	-0.035	0.131	0.2587
Serum Albumin (g/dl)	-0.700	-1.136	-0.263	0.0019
Serum TIBC (µg/dl)	0.001	-0.002	0.003	0.6179

R squared = 0.3049

Model F

	Coefficient	95% CI		p value
Intercept	2.250	0.337	4.163	0.0215
GFR (MDRD) (ml/min/1.7m^2)	-0.016	-0.022	-0.010	0.0000
UACR (mg/g)	0.081	0.005	0.156	0.0359
Serum Phosphorus (mg/dl)	0.195	-0.055	0.445	0.1251
Serum Alkaline phosphatase (IU/l)	0.370	-0.021	0.761	0.0638

R squared = 0.2865

Model G

	Coefficient	95% CI		p value
Intercept	5.108	4.061	6.156	0.0000
Age (years)	-0.004	-0.015	0.008	0.5322
GFR (MDRD) (ml/min/1.7m^2)	-0.018	-0.025	-0.011	0.0000
UACR (mg/g)	0.000	0.000	0.001	0.0029

R squared = 0.2675

Model H

	Coefficient	95% CI		p value
Intercept	6.176	2.686	9.666	0.0006
Age (years)	-0.005	-0.017	0.007	0.4203
GFR (MDRD) (ml/min/1.7m^2)	-0.018	-0.026	-0.009	0.0000
FEMg (%)	0.014	-0.026	0.054	0.4919
Serum Calcium (mg/dl)	-0.223	-0.544	0.099	0.1728
Serum Phosphorus (mg/dl)	0.239	-0.030	0.507	0.0811
Serum Alkaline phosphatase (IU/l)	0.005	0.000	0.009	0.0495
Serum intact PTH (pg/ml)	-0.001	-0.003	0.001	0.4272

R squared = 0.2725

2. Pulse Wave Assessment cohort

The Pulse Wave Assessment cohort consisted of 191 subjects. Most of them were diabetic (153/191, 80%) and had CKD (153/191, 80%). DM was the main cause of the CKD (134/191, 70%). This cohort included 38/191 (20%) non-CKD patients with high CV risk. Most CKD patients were in CKD stage 3 (80/191, 42%). Mean age was 61.2±12.9 years and most of the patients were males (140/191, 73%). This population was borderline obese with mean BMI 29.6±5.2 kg/m^2 and cfPWV was above normal levels for age in 85/191 (44.5%).

Main patient characteristics (Table 19) and pulse wave characteristics (Table 20) were summarized below.

Table 19: Main characteristics of patients in the pulse wave assessment cohort (n=191)

Total patients n (%)		191 (100)
DM n (%)		153 (80.1)
Hypertension n (%)		167 (87.4)
CVD n (%)		41 (21.5)
CKD Etiology n (%)	Non CKD (control)	38 (19.9)
	DM	134 (70.2)
	Vascular	16 (8.4)
	Glomerulonephritis	1 (0.5)
	Unknown	2 (1)
CKD Stages n (%)	Non CKD (control)	38 (19.9)
	Stage 1	16 (8.4)
	Stage 2	49 (25.7)
	Stage 3A	47 (24.6)
	Stage 3B	33 (17.3)
	Stage 4	7 (3.7)
	Stage 5	1 (0.5)
Cigarette smoking n (%)	Non smoker	77 (40)
	Active smoker	49 (26)
	Ex-smoker	65 (34)
Alcohol consumption n (%)	Non consumer	126 (66)
	Active consumer	57 (30)
	Ex-consumer	8 (4)
Age (years)		61.2±12.9
Gender n (%)	Males	140 (73)
	Females	51 (27)
Height (cm)		168.0±9.4
Weight (kg)		83.8±17.1
Body Mass Index (kg/m^2)		29.6±5.2

Table 20: Pulse Wave characteristics of patients in the pulse wave assessment cohort (n=191)

Variable		Value
Systolic Blood Pressure (mmHg)		141.2±18.8
Diastolic Blood Pressure (mmHg)		78.7±12.9
Mean Blood Pressure (mmHg)		103.1±14.0
Pulse Pressure (mmHg)		62.5±16.8
Mean PWV (m/sec)		10.9±3.1
SD PWV (m/sec)		1.2±0.7
CF-PTT (SD) (%)		10.5±4.4
PWV Reference Range n (%)	within normal	92 (48.2)
	high normal	14 (7.3)
	above normal	85 (44.5)
Normal values for age (m/sec)		12.0±2.4
Delta PWV (m/sec)		0.8±1.6
Aortic Systolic pressure (mmHg)		139.9±19.3
Aortic Pulse Pressure(mmHg)		60.9±17.6
Aortic AIx@75 (%)		24.6±18.7
SEVR (%)		141.1±30.0

Mean eGFR was 64±21.7 ml/min/1.73m^2 and the median (IQR) UACR was 97.2 (23.3, 348). Regarding the UACR, 68 patients (35.6%), 72 patients (37.7%), 33 patients (17.3%) and 18 patients (9.4%) had UACR of < 30, 30 to 299, 300 to 1000 and > 1000 mg /g respectively.

The baseline urinalysis parameters (Table 21), distribution of albuminuria (Table 22), serum analysis parameters (Table 23) and echocardiograms (Table 24) for the pulse wave assessment cohort were summarized below.

Table 21: Urinalysis parameters of the pulse wave assessment cohort (n=191)

Urinalysis Parameters / (Number of patients)	Pulse Wave Assessment cohort
Total patients	191
Creatinine Clearance (ml/min) / (158)	74.1±33.6
Diuresis (ml/24h) / (158)	1894.0±584.8
Total Proteinuria (mg/24h) / (153)	275 (137, 670)
Glycosuria (mg/dl) / (167)	81.8 ± 230.2
Spot Proteinuria (mg/dl) / (157)	17.7 (8.2, 36.5)
UPCR (mg/g) / (156)	240.3 (120.0, 562.3)
Total Microalbuminuria (mg/24h) / (54)	152.4 (38.4, 336.0)
UACR (mg/g) / (175)	97.2 (23.3, 348.0)
Spot Creatinuria (mg/dl) / (175)	85.7±52.1
Urinary Sodium (mmol/L) / (146)	91.9±38.3
Urinary Potassium (mmol/L) / (146)	37.3±14.6
Urinary Magnesium (mg/dl) / (130)	4.1±2.2
Urinary Magnesium (mg/24h) / (130)	71.7±34.6
Urinary Magnesium (mg/g Cr.) / (130)	0.059±0.030
FEMg (%) / (130)	5.6±3.2
Urinary Calcium (mg/dl) / (132)	4.9±4.5
Phosphaturia (mg/dl) / (131)	35.8±14.9
Phosphaturia (mg/24h) / (131)	644.7±256.1
Phosphaturia (mg/mg Cr.) / (131)	0.514±0.160

Table 22: Distribution of UACR among the pulse wave assessment cohort (n=191)

UACR (mg/g)	Number of patients (%)
<30	68 (35.6)
30-299	72 (37.7)
300-1000	33 (17.3)
>1000	18 (9.4)
Total	191 (100)

Table 23: Serum analysis parameters of the pulse wave assessment cohort (n=191)

Laboratory Parameters / (Number of patients)	Pulse Wave Assessment cohort
Total patients	191
Serum Creatinine (mg/dl) / (191)	1.2±0.5
GFR (MDRD) (ml/min/1.73 m^2) / (191)	64.0±21.7
Serum Glucose (mg/dl) / (140)	140.2±56.5
Serum Uric Acid (mg/dl) / (185)	6.6±1.8
Serum HbA1C (%) / (185)	7.3±1.5
Haemoglobin (g/dl) / (191)	14.0±1.6
Serum Albumin (g/dl) / (179)	4.3±0.3
hsCRP (mg/dl) / (90)	0.8±1.8
Serum Prealbumin (mg/dl) / (118)	28.9±6.8
Serum Transferrin (mg/dl) / (177)	255.4±50.1
Serum Ferritin (ng/ml) / (182)	109.5 (53.3, 192.8)
Serum Total Cholesterol (mg/dl) / (191)	163.1±37.5
Serum LDL Cholesterol (mg/dl) / (188)	89.6±30.1
Serum HDL Cholesterol (mg/dl) / (188)	44.3±13.2
Serum Triglycerides (mg/dl) / (191)	147.6±89.1
Plasma Renin (ng/ml/hr) / (98)	2.95 (1.30, 7.20)
Plasma Aldosterone (pg/ml) / (111)	91.0 (64.6, 129.5)
Serum CO_2 (mEq/L) / (145)	28.0±3.4
Serum LDH (IU/l) / (188)	374.5±89.9
Serum Sodium (mmol/l) / (189)	140.2±2.7
Serum Potassium (mmol/l) / (189)	4.6±0.5
Serum Magnesium (mg/dl) / (148)	1.9±0.2
Serum Calcium (mg/dl) / (184)	9.5±0.4
Serum Phosphorus (mg/dl) / (180)	3.4±0.6
Serum Alkaline Phosphatase (IU/l) / (187)	77.9±25.3
Serum Iron (εg/dl) / (183)	75.8±26.5
Serum TIBC (εg/dl) / (182)	325.4±60.3
Serum intact PTH (pg/ml) / (164)	51.6 (38.1, 87.1)
Serum 25(OH)D (ng/ml) / (164)	20.2±10.5
Serum $1,25(OH)_2D$ (pg/ml) / (66)	34.9±14.4
Serum Vitamin B12 (pg/ml) / (167)	416.7±156.3
Serum Folic Acid (ng/ml) / (159)	8.8±4.3
Serum TSH (εIU/ml) / (178)	2.4±1.4
Serum Free T3 (pg/ml) / (133)	3.1±0.5
Serum Free T4 (ng/dl) / (153)	1.2±0.2
Serum Vitamin A (mg/l) / (96)	0.7±0.2
Serum Vitamin E (εg/ml) / (96)	14.2±4.3
Plasma PCSK9 (ng/ml) / (54)	296.0±102.1
Plasma CXCL16 (ng/ml) / (54)	3.9±0.8

Table 24: Echocardiograms for pulse wave assessment cohorts (n=191)

Variable		Value
Total Patients n (%)		140 (100)
Ejection Fraction (%)		59.9±6.0
Left Ventricular Diameter (mm)		43.2±4.8
Interventricular septum (mm)		10.2±1.4
Left Ventricular Hypertrophy n (%)		56 (40)
Altered Relaxation n (%)		91 (65)
Cardiomyopathy n (%)	None	126 (87.1)
	Hypertrophic	16 (11.4)
	Dilated	2 (1.4)
Valvular Calcification n (%)		23 (16.4)
Pericardial Effusion n (%)		3 (2.1)

The majority of the patients were receiving antidiabetic; oral hypoglycemic agents; 90/191, 47% and Insulin; 100/191, 52%) or antihypertensive (168/191, 88%) medications. Lipid lowering therapy was prescribed to 145/191 (76%). Over 50 % of the patients were taking anti-platelet therapy. A minority of patients were receiving vitamin D supplementation (49/191, 26%), phosphate binders (9/191, 5%), or ESAs (3/191, 2%). Patientsô medications (Table 25) and dosage for selected medications (Table 26) were summarizes below.

Table 25: Medications of the patients in the Pulse Wave Assessment cohort; only patients received the medications were analyzed.

Medication	Number of patients (%)
Total patients	191 (100)
Any vitamin D or VDR activator	49 (25.7)
Paricalcitol (mcg/w)	17 (8.9)
Calcifediol (IU/w)	37 (19.4)
Calcitriol (mcg/w)	3 (1.6)
Cinacalcet (mg/w)	0
Any phosphate binder	9 (4.7)
Lanthanum	1 (0.5)
Sevelamer	1 (0.5)
Aluminium- based phosphate binders	1 (0.5)
Calcium-based phosphate binders	6 (3.1)
Calcium Supplement	3 (1.6)
Calcium Polystyrene Sulfonate (Potassium chelator)	9 (4.7)
Iron supplement	23 (12)
ESAs	3 (1.6)
Oral hypoglycemic agents	90 (47.1)
Insulin	100 (52.4)
Any lipid lowering agent	145 (75.9)
Statin	132 (69.1)
Fibrate	28 (14.7)
Ezetimibe	16 (8.4)
Omega 3-Fatty Acids	15 (7.9)
Any anti-hypertensive *	168 (88)
Any RAAS blocker	166 (86.9)
ACEIs	84 (44)
ARBs	103 (53.9)
Spironolactone	11 (5.8)
Calcium Channel Blockers	97 (50.8)
Beta Blockers	45 (23.6)
Alpha Blockers	35 (18.3)
Alpha & Beta Blockers	2 (1)
Diuretics	107 (56)
Proton Pump Inhibitors	69 (36.1)
Anti-platelets	101 (52.9)

* Including RAAS blockers, calcium channel blockers, beta blockers, alpha & beta blockers and diuretics

Table 26: Dosage for selected medications; only patients received the medications were analyzed.

Medication	Mean±SD or Median (IQR)
Paricalcitol (mcg/w)	4.1±2.1
Calcifediol (UI/w)	2160 (1440, 4000)
Calcitriol (mcg/w)	0.6±0.3

Mean Pulse Wave Velocity

In Pulse Wave Assessment cohort the mean±SD of the PWV were 10.9±3.1 m/sec. In the whole cohort 85/191 (44.5%) of patients had mean PWV above normal values for their age (Table 17). Among CKD patients 73/153 (47.7%) had mean PWV above normal values for their age, with mean PWV 13.6±2.6 m/sec. Among non-CKD patients 12/38 (31.6%) had mean PWV above normal values for their age, with mean PWV 10.8±2.3 m/sec.

We explored the association of mean PWV with clinical, echocardiogram, therapeutic and laboratory parameters.

The univariate analysis of mean PWV (m/sec) with other quantitative variables (Table 27) and qualitative variables (Table 28), showed a significant positive correlations between mean PWV and DM, hypertension, history of CVD, male gender and treatment with calcium containing phosphate binders, resin calcium, iron supplement, statin, ezetimibe, ARBs, diuretics, alpha blockers, proton pump inhibitors, anti-platelets. In addition, PWV positively correlated with age, systolic BP, pulse pressure, ejection fraction, UACR, serum uric acid, glucose and HbA1c. Also mean PWV was significantly negatively correlated with GFR, total serum cholesterol, LDL cholesterol, $1,25(OH)_2D$ and SEVR (Figures 21). Mean PWV had a trend towards a positive correlation with altered ventricular relaxation and serum potassium, while there was a trend towards a negative correlation with serum 25(OH)D and free T3

Table 27: Correlations between mean PWV (m/sec) and quantitative variables in univariate analysis. Only statistically significant ($p < 0.05$) results or trends ($p \geq 0.05$ to $p < 0.1$)] are shown.

Variable	N	Coefficient	P value
Age (years)	191	0.4533	0.0000
Systolic Blood Pressure (mmHg)	191	0.4207	0.0000
Pulse Pressure (mmHg)	191	0.4692	0.0000
Ejection Fraction (%)	140	0.1964	0.0201
GFR (MDRD) (ml/min/1.73 m^2)	190	-0.2890	0.0001
UACR (mg/g)	175	0.1574	0.0375
Serum uric acid (mg/dl)	185	0.2928	0.0001
Serum glucose (mg/dl)	190	0.1542	0.0336
HbA1c (%)	185	0.2588	0.0004
Serum Total cholesterol (mg/dl)	190	-0.2281	0.0015
Serum LDL cholesterol (mg/dl)	188	-0.2387	0.0010
Serum Potassium (mmol/l)	189	0.1425	0.0504
Serum 25(OH)D (ng/ml)	164	-0.1347	0.0854
Serum 1,25 $(OH)_2D$ (pg/ml)	66	-0.3363	0.0058
Serum Free T3 (pg/ml)	133	-0.1703	0.0500
SEVR or Buckberg Index (%)	183	-0.3742	0.0000

Table 28: Correlations between mean PWV (m/sec) and qualitative variables in univariate analysis. Only statistically significant ($p < 0.05$) results or trends ($p \geq 0.05$ to $p < 0.1$)] are shown.

Variable	N	Mean	SD	P value
DM				
No	38	8.74	2.19	
Yes	153	11.47	3.01	0.0000
HTN				
No	24	8.36	2.41	
Yes	167	11.3	2.97	0.0000
CVD				
No	150	10.66	2.94	
Yes	41	11.91	3.33	0.0199
Gender				
Females	51	9.95	2.85	
Males	140	11.29	3.07	0.0072
Calcium-Based Phosphate Binders				
No	185	10.83	3	
Yes	6	13.92	3.6	0.0147
Calcium polystyrene sulfonate				
No	182	10.72	2.93	
Yes	9	15.11	2.65	0.0000
Iron supplement				
No	168	10.73	2.93	
Yes	23	12.37	3.67	0.0160
Statin				
No	59	9.98	2.79	
Yes	132	11.35	3.09	0.0040
Ezetimibe				
No	175	10.78	3.04	
Yes	16	12.6	2.89	0.0221
ARBs				
No	88	10.07	2.73	
Yes	103	11.67	3.15	0.0003
Alpha blockers				
No	156	10.69	2.98	
Yes	35	12.01	3.21	0.0202
Diuretics				
No	84	10.2	2.88	
Yes	107	11.5	3.09	0.0032
Proton Pump Inhibitors				
No	122	10.39	2.81	
Yes	69	11.88	3.27	0.0011
Anti-Platelets				
No	90	9.88	2.79	
Yes	101	11.87	3.00	0.0000
Altered ventricular relaxation				
No	49	10.69	2.64	
Yes	91	11.65	3.29	0.0794

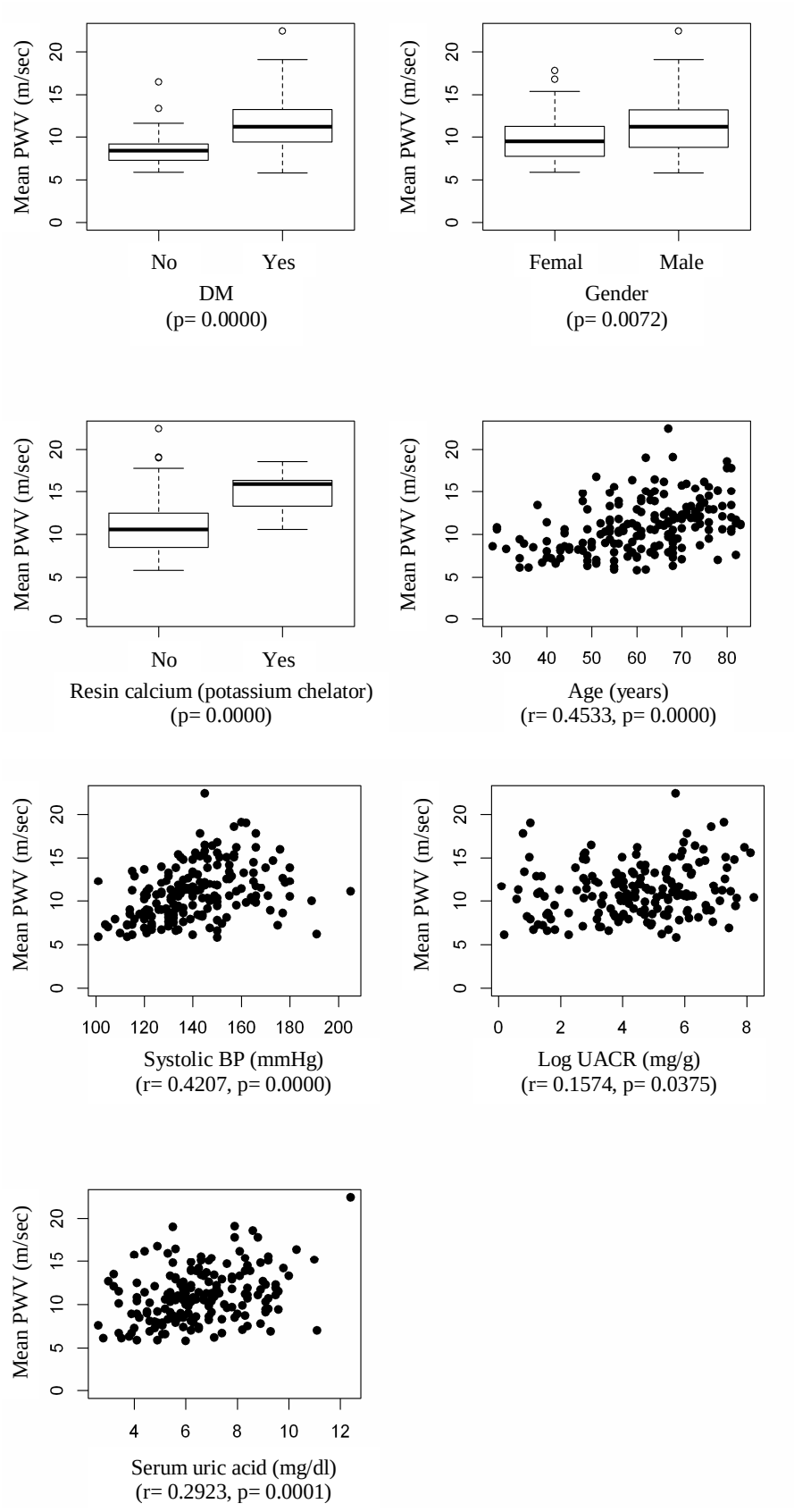


Figure 21: Statistically significant correlations with Mean PWV

Different multivariate models (Table 29) showed that advanced age, systolic BP, DM, serum uric acid, UACR and resin calcium therapy were independently positively correlated with the mean PWV. The best R squared obtained was 0.335.

Table 29: Different multivariate models for predictors of mean PWV (m/sec)

Model A

	Coefficient	95% CI		p value
Intercept	-1.015	-4.156	2.125	0.5243
DM	1.287	0.296	2.279	0.0112
Age (years)	0.062	0.031	0.092	0.0001
Serum Uric acid (mg/dl)	0.288	0.076	0.501	0.0080
Systolic Blood Pressure (mmHg)	0.036	0.016	0.057	0.0006
Calcium polystyrene sulfonate	2.968	1.258	4.679	0.0008

R squared= 0.3354

Model B

	Coefficient	95% CI		p value
Intercept	-0.209	-3.775	3.357	0.9079
Gender (male)	1.043	0.197	1.890	0.0160
DM	1.250	0.251	2.250	0.0145
Age (years)	0.066	0.033	0.099	0.0001
Systolic Blood Pressure (mmHg)	0.026	-0.005	0.057	0.0948
Pulse Pressure (mmHg)	0.026	-0.012	0.065	0.1732

R squared = 0.3109

Model C

	Coefficient	95% CI		p value
Intercept	-1.618	-4.830	1.595	0.3217
DM	1.380	0.361	2.399	0.0082
Age (years)	0.070	0.039	0.101	0.0000
Serum Uric Acid (mg/dl)	0.309	0.091	0.528	0.0057
Systolic Blood Pressure (mmHg)	0.036	0.015	0.057	0.0008

R squared = 0.2958

Model D

	Coefficient	95% CI		p value
Intercept	4.627	1.465	7.790	0.0044
DM	1.101	-0.162	2.364	0.0870
CVD	-0.134	-1.134	0.867	0.7925
Gender (male)	0.784	-0.157	1.724	0.1017
Age (years)	0.086	0.050	0.123	0.0000
GFR (MDRD) (ml/min/1.73 m^2)	-0.008	-0.029	0.013	0.4332
UACR (mg/g)	0.001	0.000	0.001	0.0348

R squared = 0.2036

Delta above upper limit of normal PWV (Delta PWV)

Since the PWV increases with age and indeed we found a very significant correlation between PWV and age (Figure 22), we next explored parameters associated with the absolute increase in PWV over the higher expected normal limit of the age-adjusted PWV in the general population. Thus, the Delta PWV parameter was calculated as follows= (measured PWV) - (Upper limit of the age-adjusted PWV values for the general population).

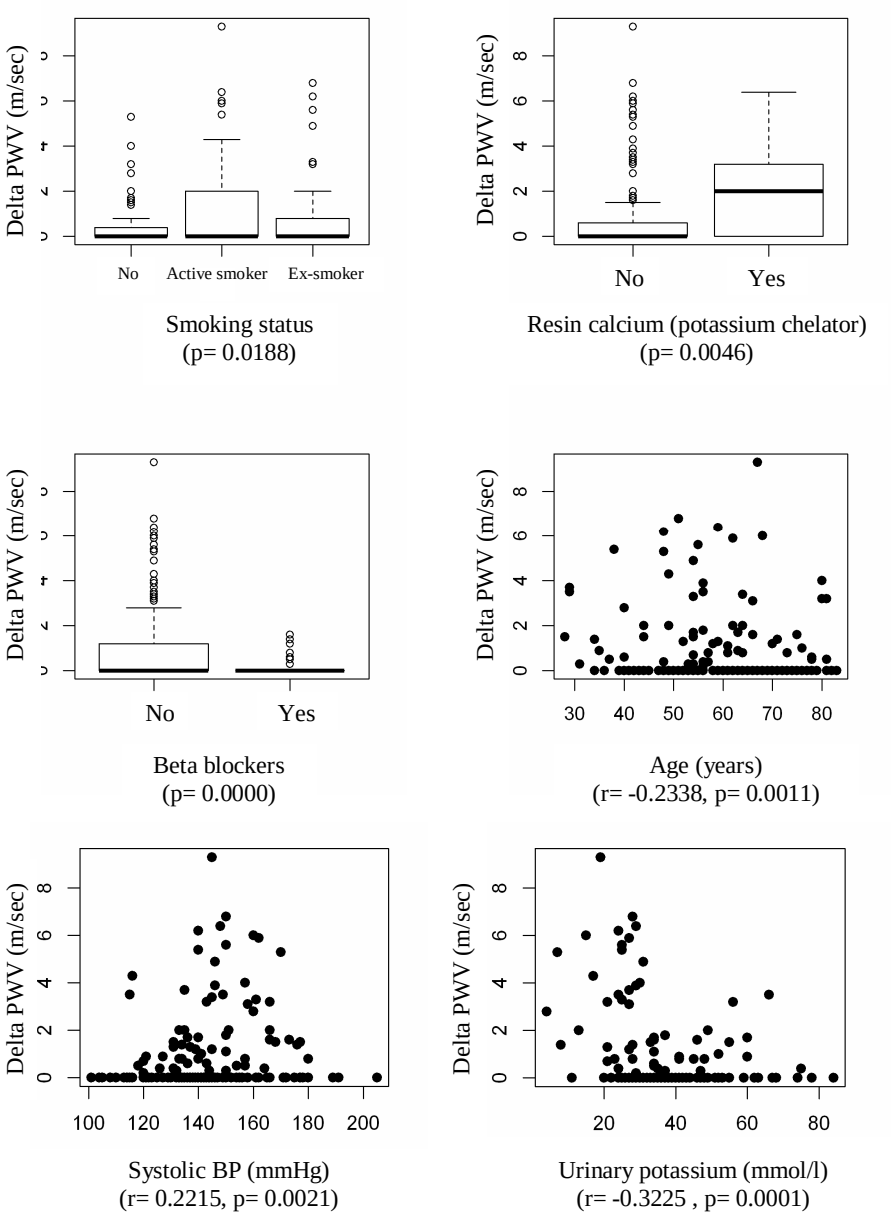


Figure 22: Statistically significant correlations with Delta PWV

The mean±SD of the Delta PWV were 0.8±1.6 m/sec. That is, as a mean, the PWV was 0.8 m/sec higher than the expected PWV for the patientôs age. We explored the association of delta PWV with clinical, echocardiogram, therapeutic and laboratory parameters.

The univariate analysis of delta PWV with other qualitative (Table 30) and quantitative (Table 31) variables, showed that delta PWV was significantly positively correlated with DM, active smoking and use of resin calcium or ARBs. In addition, delta PWV was significantly positively correlated with systolic BP, diastolic BP, mean BP, UACR and HbA1c. On the other hand, delta PWV had significant negative correlations with age, SEVR, valvular calcification, use of calcium supplementation or beta blockers, serum CO_2, serum sodium, urinary potassium and phosphaturia. The delta PWV had a trend towards a positive correlation with serum phosphorus and plasma CXCL16, and a trend towards a negative correlation with serum free T4 and creatinuria.

Table 30: Correlations between delta PWV (m/sec) and qualitative variables in univariate analysis. Only statistically significant results ($p < 0.05$) or trends ($p \geq 0.05$ to $p < 0.1$)] are shown.

Variable	N	Mean	SD	P value
DM				
No	38	0.27	0.68	
Yes	153	0.89	1.74	0.0008
Smoking				
Non-smoker	77	0.43	0.96	
Active smoker	49	1.36	2.25	
Ex-smoker	65	0.72	1.53	0.0188
Valvular calcification				
No	117	0.91	1.82	
Yes	23	0.38	0.63	0.0148
Pericardial effusion				
No	137	0.84	1.71	
Yes	3	0.07	0.12	0.0000
Calcium supplement				
No	188	0.78	1.61	
Yes	3	0.00	0.00	0.0000
Calcium polystyrene sulfonate				
No	182	0.69	1.54	
Yes	9	2.23	2.18	0.0046
ARBs				
No	88	0.45	1.08	
Yes	103	1.03	1.91	0.0087
Beta blockers				
No	146	0.95	1.78	
Yes	45	0.18	0.40	0.0000

Table 31: Correlations between delta PWV (m/sec) and quantitative variables in univariate analysis. Only statistically significant results ($p < 0.05$) or trends ($p \geq 0.05$ to $p < 0.1$)] are shown.

Variable	N	Coefficient	P value
Age (years)	191	- 0.2338	0.0011
Systolic Blood Pressure (mmHg)	191	0.2215	0.0021
Diastolic Blood Pressure (mmHg)	191	0.1649	0.0226
Mean Blood Pressure (mmHg)	191	0.2004	0.0054
SEVR or Buckberg index (%)	183	- 0.2874	0.0001
UACR (mg/g)	175	0.1774	0.0188
Urinary Creatinine (mg/dl)	175	- 0.1399	0.0648
HbA1c (%)	185	0.1667	0.0233
Serum CO_2 (mEq/l)	145	- 0.1986	0.0166
Serum sodium (mmol/l)	189	- 0.1557	0.0324
Serum phosphorus (mmol/l)	180	0.1395	0.0618
Urinary phosphorus (mg/dl)	131	- 0.1812	0.0384
Urinary potassium (mmol/l)	146	- 0.3225	0.0001
Serum Free T4 (mg/dl)	153	- 0.1551	0.0556
Plasma CXCL16 (ng/ml)	54	0.2376	0.0836

In the multivariate analysis (Table 32), systolic BP, active smoking and resin calcium therapy remained independently positively correlated with delta PWV, while age, urinary potassium and beta blocker therapy were independently negatively correlated with delta PWV. The multivariate model explain a little of the delta PWV variability with R squared of 0.27.

Table 32: Multivariate analysis model for predictors of delta PWV (m/sec)

	Coefficient	95% CI		p value
Intercept	0.970	- 1.679	3618	0.4703
DM	0.437	- 0.844	1.718	0.5007
Gender (Male)	0.023	- 0.626	0.672	0.9442
Age (years)	- 0.038	0.060	- 0.015	0.0014
Smoking				
Active smoker	1.036	0.331	1.742	
Ex-smoker	0.409	- 0.234	1.053	0.0152
Calcium polystyrene sulfonate	1.278	0.193	2.362	0.0212
Beta blockers	- 0.971	- 1.554	- 0.388	0.0013
Systolic Blood Pressure (mmHg)	0.019	0.005	0.033	0.0092
Urinary potassium (mmol/l)	- 0.029	- 0.047	- 0.011	0.0015

R squared = 0.2729

Aortic Augmentation Index adjusted by heart rate (Aortic AIX@75)

The mean±SD of the Aortic AIX@75 was 24.6±18.7 %. We explored the association of Aortic AIX@75 with clinical, echocardiogram, therapeutic and laboratory parameters.

The univariate analysis of the Aortic AIX@75 with other qualitative (Table 33) and quantitative (Table 34) variables, showed significant positive correlations between Aortic AIX@75 and systolic BP, Mean BP, pulse pressure, UACR and therapy with statin, beta blockers, anti-platelets or calcifediol. Aortic AIX@75 had significant negative correlations with male gender, height, weight and ejection fraction (Figure 23). A trend towards a positive correlation was found between Aortic AIX@75 and CVD, diuretic therapy and serum sodium and LDH, while a trend towards a negative correlation was found with paricalcitol therapy, serum magnesium and Hb.

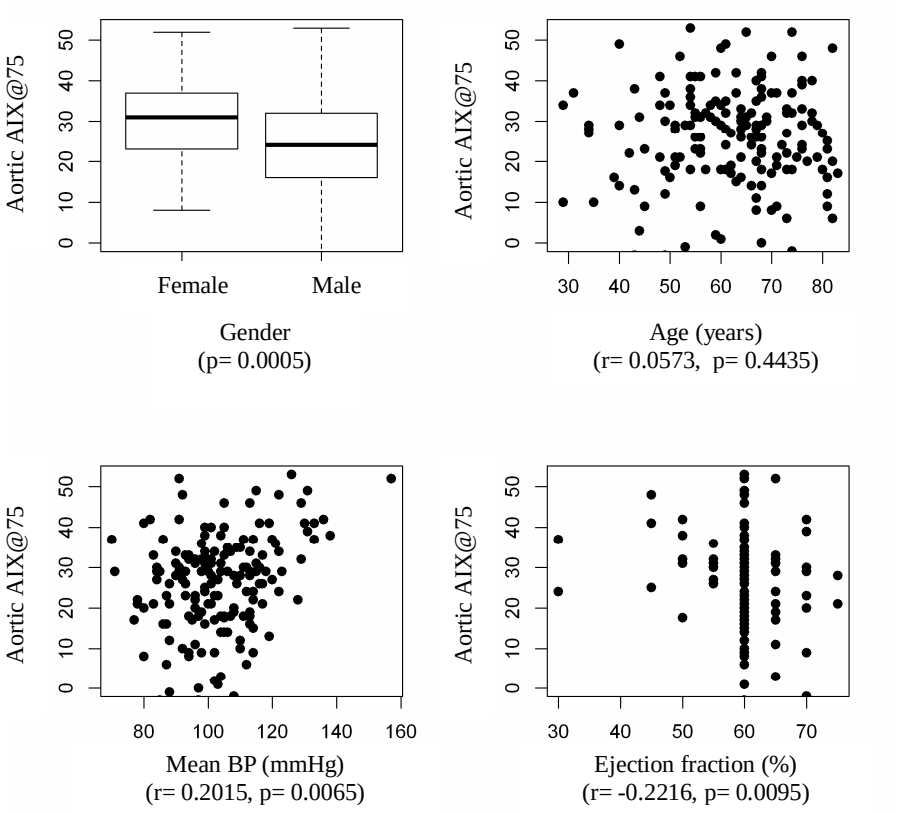


Figure 23: Statistically Significant Correlations with Aortic AIX@75

Table 33: Correlations between Aortic AIX@75 (%) and qualitative variables in univariate analysis. Only statistically significant results ($p < 0.05$) or trends ($p \geq 0.05$ to $p < 0.1$)] are shown.

Variable	N	Mean	SD	P value
CVD				
No	142	22.81	15.24	
Yes	39	27.25	13.15	0.0995
Gender				
Females	47	30.15	12.73	
Males	134	21.53	14.99	0.0005
Oral hypoglycemic agent				
No	92	24.48	14.62	
1 drug	48	20.06	15.11	
2 drugs	25	31.64	9.63	0.0060
3 drugs	16	18.48	17.89	
Statin				
No	54	20.39	16.99	
Yes	127	25.20	13.73	0.0464
Beta blockers				
No	138	22.14	15.72	
Yes	43	28.98	10.36	0.0013
Diuretics				
No	78	21.19	16.88	
Yes	103	25.71	12.93	0.0510
Anti-Platelets				
No	83	20.88	16.82	
Yes	98	26.21	12.61	0.0188
Calcifediol				
No	146	20.20	15.30	
Yes	35	30.31	10.98	0.0006

Table 34: Correlations between Aortic AIX@75 (%) and quantitative variables in univariate analysis. Only statistically significant results ($p < 0.05$) or trends ($p \geq 0.05$ to $p < 0.1$)] are shown.

Variable	N	Coefficient	P value
Height (cm)	181	- 0.2637	0.0003
Weight (kg)	181	- 0.2340	0.0015
Systolic Blood Pressure (mmHg)	181	0.1847	0.0128
Mean Blood Pressure (mmHg)	181	0.2015	0.0065
Pulse Pressure (mmHg)	181	0.1576	0.0341
Ejection Fraction (%)	136	- 0.2216	0.0095
Paricalcitol dosage (mcg/w)	181	- 0.1279	0.0862
Calcifediol dosage (UI/w)	181	0.2124	0.0041
UACR (mg/g)	168	0.2377	0.0019
Haemoglobin (g/dl)	180	- 0.1300	0.0821
Serum LDH (IU/l)	179	0.1280	0.0877
Serum Sodium (mmol/l)	180	0.1241	0.0969
Serum magnesium (mg/dl)	142	- 0.1612	0.0553

In the multivariate analysis (Table 35). , mean BP and age independently positively correlated with Aortic AIX@75, while male gender and ejection fraction were independently negatively correlated with Aortic AIX@75. Age was added to the multivariate analysis despite not being significant in the univariate analysis because of its strong effect on arterial stiffness. The multivariate model explain a little of the Aortic AIX@75 variability with R squared of 0.20.

Table 35: Multivariate regression analysis for predictors of Aortic AIX@75

	Coefficient	95% CI		p value
Intercept	12.002	-20.703	44.706	0.4690
Age (years)	0.246	0.047	0.444	0.0156
Gender (Male)	-11.759	-17.308	-6.211	0.0001
Mean Blood Pressure (mmHg)	0.311	0.135	0.488	0.0007
Ejection Fraction (%)	-0.517	-0.892	-0.142	0.0073
UACR (mg/g)	1.028	-0.247	2.303	0.1130

R squared = 0.2252

Subendocardial viability ratio (SEVR; or Buckberg Index)

The mean±SD of the SEVR was 141.1±30.0 %. We explored the association of SEVR with clinical, echocardiogram, therapeutic and laboratory parameters.

The univariate analysis of SEVR with other qualitative (Table 36) and quantitative (Table 37) variables, showed significant positive correlations between SEVR and male gender, beta blockers therapy as well as between SEVR and serum Hb, sodium and 25(OH)D. SEVR had significant negative correlations with DM, systolic BP, mean BP, pulse pressure or therapy with iron supplementation, ezetimibe or proton pump inhibitors. In addition, SEVR had significant negative correlations with mean PWV, ejection fraction, serum glucose, HbA1c, hsCRP, LDH, potassium, phosphorus and vitamin B12 (Figure 24). A trend towards a positive correlation was found between SEVR and serum CO_2 and free T3, while a trend towards a negative correlation was found with plasma CXCL16 and serum TIBC.

Table 36: Correlations between Subendocardial Viability Ratio (SEVR; Buckberg Index) (%) and qualitative variables in univariate analysis. Only statistically significant results ($p < 0.05$) or trends ($p \geq 0.05$ to $p < 0.1$) are shown .

Variable	N	Mean	SD	P value
DM				
No	35	150.37	25.72	
Yes	148	138.93	26.87	0.0236
Gender				
Females	47	129.21	24.03	
Males	136	145.24	26.78	0.0004
Iron Supplement				
No	160	143.16	26.93	
Yes	23	126.96	23.10	0.0067
Ezetimibe				
No	168	142.41	26.93	
Yes	15	126.67	23.58	0.0299
Beta blockers				
No	139	138.65	25.05	
Yes	44	148.93	31.33	0.0271
Proton Pump Inhibitors				
No	115	144.72	29.04	
Yes	68	135.03	21.91	0.0115

Table 37: Correlations between Subendocardial Viability Ratio (SEVR; Buckberg Index) (%) and quantitative variables in univariate analysis. Only statistically significant results ($p < 0.05$) or trends ($p \geq 0.05$ to $p < 0.1$) are shown.

Variable	N	Coefficient	P value
Systolic Blood Pressure (mmHg)	183	- 0.2897	0.0001
Mean Blood Pressure (mmHg)	183	- 0.1488	0.0444
Pulse Pressure (mmHg)	183	- 0.3174	0.0000
Mean PWV (m/sec)	183	- 0.3742	0.0000
Ejection Fraction (%)	135	- 0.2025	0.0185
Serum glucose (mg/dl)	182	- 0.1872	0.0114
HbA1c (%)	178	- 0.2045	0.0062
Haemoglobin (g/dl)	182	0.1827	0.0136
hsCRP (mg/dl)	84	- 0.2618	0.0162
Serum LDH (IU/l)	181	- 0.1799	0.0154
Serum Sodium (mmol/l)	182	0.1634	0.0275
Serum potassium (mmol/l)	182	- 0.1682	0.0233
Serum phosphorus (mg/dl)	173	- 0.1539	0.0432
Serum 25 (OH) Vitamin-D (ng/ml)	159	0.3136	0.0001
Serum Vitamin-B12 (pg/ml)	160	- 0.1560	0.0489
Serum Free T3 (pg/ml)	128	0.1583	0.0742
Plasma CXCL16 (ng/ml)	54	- 0.2487	0.0697
Serum CO_2 (mEq/l)	140	0.1513	0.0743
Serum TIBC (µg/dl)	176	- 0.1412	0.0615

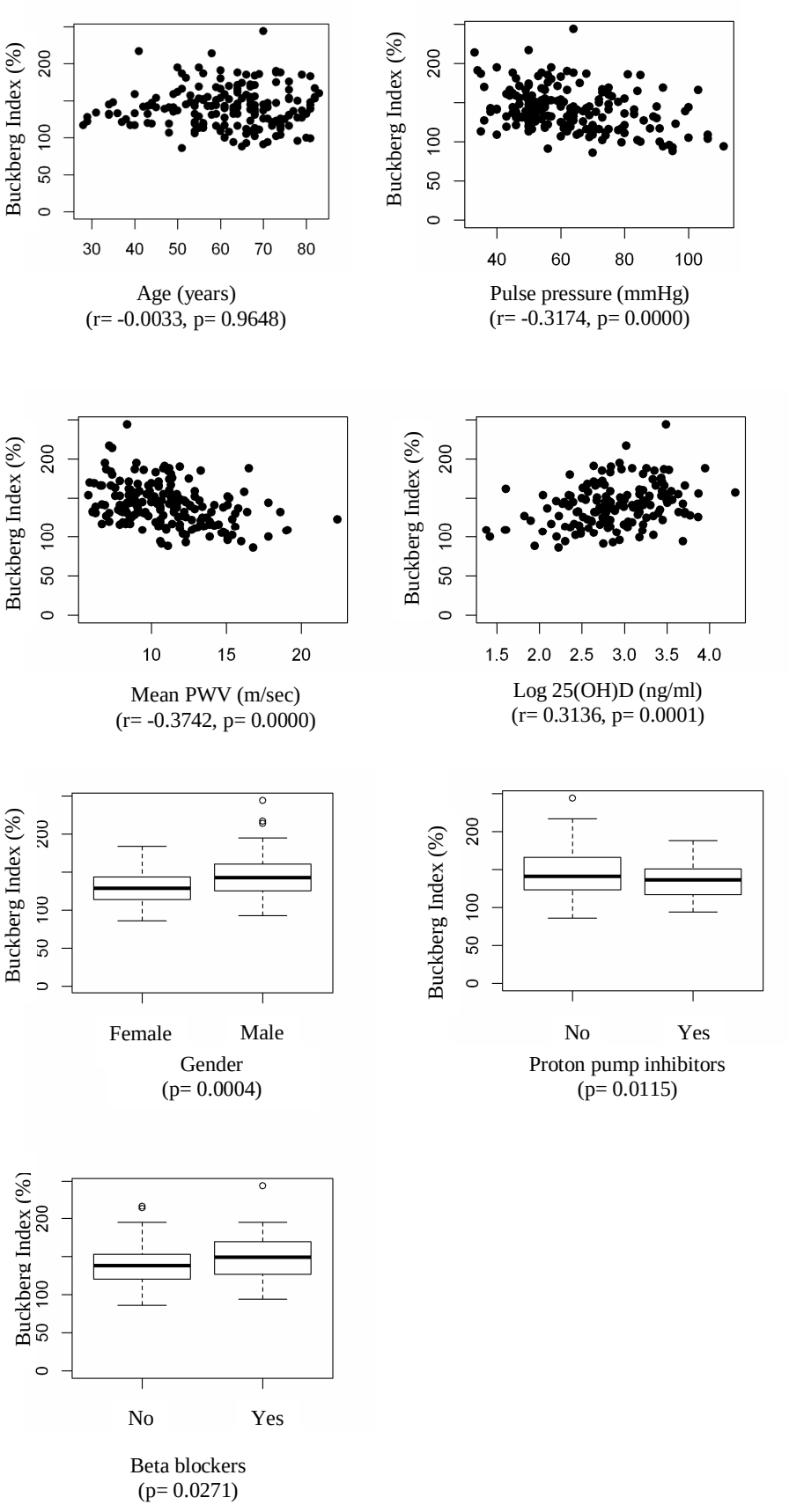


Figure 24: Statistically Significant Correlations with SEVR or Buckberg Index

In the multivariate analysis (Table 38), beta blocker therapy and log 25(OH)D independently positively correlated with SEVR, while age, female gender, pulse pressure, mean PWV and proton pump inhibitor therapy were independently negatively correlated with SEVR. Age was added to the multivariate analysis despite not being significant in the univariate analysis because of the strong effect of advanced age on arterial stiffness and cardiac performance. The multivariate model explains little of the SEVR variability with the highest R squared obtained being 0.39.

Table 38: Multivariate regression analysis for predictors of Subendocardial Viability Ratio [SEVR] (%)

	Coefficient	95% CI		p value
Intercept	129.4	100.7	158.0	0.0000
Age (years)	0.522	0.191	0.853	0.0022
Gender (Male)	16.58	8.58	24.57	0.0001
Pulse Pressure (mmHg)	-0.389	-0.633	-0.146	0.0019
Mean PWV (m/sec)	-3.295	-4.631	-1.958	0.0000
Log 25 (OH) Vitamin-D 25	10.21	3.62	16.81	0.0026
Proton Pump Inhibitor	-8.155	-15.650	-0.662	0.0331
Beta Blockers	8.43	0.080	16.78	0.0478

R squared = 0.3898

DISCUSSION

The main findings from this study may be summarized as follows:

1. Plasma PCSK9 values in diabetic CKD patients are very variable and the factors underlying this variability are unclear. However, the use of lipid lowering therapies containing fibrates appears to be a key contributor. We may speculate that increased PCSK9 levels may contribute to "scape" from the lipid lowering effect of lipid lowering therapies. No effects of renal function or albuminuria on PCSK9 levels were noted. Despite PCSK9 been a potential determinant of serum cholesterol levels, no relationship to cardiovascular disease was observed in this cohort.

2. By contrast to PCSK9, plasma CXCL16 correlated with renal function and albuminuria as well as with parameters of bone mineral metabolism. The present observational study does not provide clues as to cause-effect relationships. Increased CXCL16 levels may represent an aspect of the systemic inflammation associated with uremia. Alternatively, higher CXCL16 levels may contribute to kidney injury or the presentation of CKD related Mineral Bone Disease (CKD-MBD). Follow-up of the cohort will provide information on the prognostic value of the assessment of this parameter. Despite inflammation been a potential contributor to cardiovascular disease, no relationship of CXCL16 with cardiovascular disease was observed in this cohort.

3. The analysis of modifiable determinants of PWV, a measure of arterial stiffness, identified serum uric acid, UACR and resin calcium therapy as independently and positively correlated with the mean PWV, while analysis of values of PWV above the age-expected normal range further identified active smoking as associated with higher delta PWV. By contrast, urinary potassium and beta blocker therapy were independently negatively correlated with delta PWV. These data identify a potential relationship between uric acid and potassium metabolism with arterial stiffness.

4. Among modifiable factors, beta blocker therapy and 25(OH)D levels independently positively correlated with subendocardial viability ratio (SEVR), suggesting that they are associated with improved myocardial perfusion. By contrast, proton pump inhibitor therapy was independently negatively correlated with SEVR.

Several aspects merit further discussion.

Plasma PCSK9

Mean plasma PCSK9 levels in diabetic CKD patients were 310±114 ng/ml. These represent relatively high levels. However, reported values in the general population and controls are very variable. Thus, previously reported mean values include 100±7 ng/ml for the general population [339], 177±13 ng/ml in 112 healthy controls [225] and 327±131 ng/ml in 178 controls [248].. These differences may relate to the type of ELISA used, and genetic or environmental factors. In our small group of 4 healthy individuals PCSK9 values were 261±76 ng/ml while in 3 non-diabetic CKD patients they were 341 ±106 ng/ml. Thus, diabetic CKD patients have PCSK9 values not very different from our small group of controls for the technique and are also similar to values reported previously for CKD patients on hemodialysis (276±110 ng/ml) for [248] and for type 2 DM patients (270±59 ng/ml) [340]. However, PCSK9 in hemodialysis patients was reported to be lower than in controls (276±110 ng/ml vs 327±131 ng/ml) [341], while Plasma PCSK9 was described as increased in proteinuric CKD subjects when compared to controls (213 [161-314] vs. 143 [113-190] ng/mL) [342].

Our regression analysis showed that the independent predictors of PCSK9 were combined statin-fibrate lipid lowering therapy, serum TIBC and plasma renin.

Lipid lowering therapy has been reported to increase plasma PCSK9 [343, 344]. This may be one of the mechanisms of resistance or poor therapy to these drugs and is specially interesting because of the expected availability of specific therapy in the near future. Recent clinical trials have reported a lipid lowering effect of monoclonal antibodies targeting PCSK9 in 160 statin-intolerant patients [345] and 631 subjects with hypercholesterolemia receiving background therapy with statin [346].

A meta-analysis indicated a significant elevation of circulating PCSK9 concentrations following fibrate therapy (95% CI: 11.04-109.71; p=0.02). The PCSK9-elevating effect of fibrates remained significant after comparison with a control group receiving either placebo or a statin (95% CI: 5.68-42.26; p=0.01). Meta-regression analysis indicated that the effect size of fibrates in modulating circulating PCSK9 levels is not dependent to the duration of treatment [343]. In an open randomized study using 400 mg bezafibrate or 200 mg fenofibrate on 14 patients with dyslipidemia with impaired glucose tolerance or type 2 DM, fibrates significantly increased plasma PCSK9 concentrations (+40% for bezafibrate and +67% for fenofibrate, p<0.001) [340]. In another study 12 weeks of fenofibrate 200 mg/day significantly increased serum PCSK9 levels by 25% in 22 patients with dyslipidemia and HbA1C<8% [339]. Fibrates stimulate a specific nuclear receptor designated as peroxisome proliferator-activated receptor alpha (PPAR-Ŭ) [341, 347, 348]. As PPAR-Ŭ agonists, fibrates improve triglyceride and HDL-Cholesterol concentrations by enhancing-oxidation of fatty acids and lipoprotein lipase (LPL) activity, increasing

production of the components of HDL (apolipoproteins A1 and A2), and reducing production of the inhibitor of LPL activity (apolipoprotein C3) [349-353], while enhancing cholesterol efflux from the liver [354]. Several reports [339, 355, 356] suggest that these effects of PPAR-Ŭon cholesterol and lipoprotein metabolism may lead indirectly to decrease hepatic intracellular cholesterol levels, and thus result in a secondary increase in PCSK9 expression and secretion. Fibrates show different binding properties against PPAR subtypes, which could cause different clinical effects on circulating PCSK9 levels [340].

Statins have also been reported to impact on PCSK9 levels. A 34% significantly higher serum PCSK9 levels were observed after 16 weeks of atorvastatin 40 mg/d [344]. In another study the 55 patients on atorvastatin and 27 patients on rosuvastatin had around 45% (P<0.001) higher plasma PCSK9 and PCSK9 increased with increasing statin dose. When atorvastatin was increased from 5 to 80 mg, plasma PCSK9 levels increased from 109±33 to 142±35 ng/ml and for rosuvastatin from 5 to 40 mg, plasma PCSK9 increased from 123±23 to 168±84 ng/ml [236]. Statins increase PCSK9 mRNA expression, and increase the activity/nuclear translocation of SREBP-2, a transcription factor that activates both the LDLR and PCSK9 genes [226, 357]. Our univariate analysis showed the significant correlations between plasma PCSK9 and statin alone and fibrate alone therapy, but in the multivariate analysis only combined fibrate-statin therapy was independent predictor which had not been reported before in the literature.

In 39 CKD patients (e-GFR 61±29 mL/min/1.73 m^2 with proteinuria 1.9 [0.9-3.3] g/day), 19 of them on statins and none on fibrates, plasma PCSK9 was elevated in proteinuria but no independent correlation with lipid lowering therapy was found [342]. However, we did not find a relationship with UACR.

There is no clear explanation for the correlation of serum TIBC with plasma PCSK9. TIBC is an indirect measure of serum transferrin concentration. Higher TIBC levels may represent iron deficiency or absence of factors that decrease transferrin, i.e. absence of inflammation or malnutrition or severe proteinuria. However, if the association between TIBC and PCSK9 represents an indirect measure of the presence or absence of any of these factors, it is unclear why no association was found with variables that more closely reflect iron status, malnutrition, inflammation or proteinuria. A common link between TIBC and PCSK9 is that they are both synthesized by hepatocytes.

There is also no clear explanation for the association between plasma PCSK9 and log plasma renin. PPAR-Ŭ may contribute to expression of renin, as knocking out the gene for PPAR-Ŭ in mice reduced plasma renin [358]. However, it is highly unlikely that fibrate use explains the association between log plasma renin and PCSK9, since the association between renin and PCSK9 remained statistically significant when combined statin-fibrate therapy was included in the multivariate model. Furthermore, mean renin activity was 13.3±28.0 ng/ml/hr in patients on fibrate and 34.9±73.3 ng/ml/hr in patients not on fibrates.

Plasma CXCL16

Mean±SD of plasma CXCL16 levels were 4.0±0.9 ng/ml. This compares with previously reported mean values of 1.30±0.05 ng/ml for the general population, 2.65±0.11 ng/ml for CKD patients, 3.04±0.16 ng/ml for DKD patients and 1.23±0.04 ng/ml for type 2 DM patients [190]. Our small group of healthy individuals had CXCL16 levels of 3.1±0.3 ng/ml. Thus, circulating CXCL16 levels appear to be increased in diabetic CKD patients.

Our multiple regression analysis demonstrated that the independent predictors of high plasma CXCL16 were low eGFR, low serum albumin, high UACR and high serum alkaline phosphatase. There was a trend towards a positive association between CXCL16 levels and serum phosphorus, while there was a trend towards a negative association with serum $1,25(OH)_2D$. Thus, a relationship was found between CXCL16 and parameters of renal function/renal injury (eGFR, UACR), nutrition (serum albumin, although there are other influences on serum albumin levels) and mineral bone metabolism (serum alkaline phosphatase, serum phosphorus, serum $1,25(OH)_2D$). The relationship with alkaline phosphatase remained significant in models that included eGFR, suggesting that this association is independent from renal function.

eGFR, serum albumin and UACR may be interrelated. Thus, there was a positive correlation between eGFR and serum albumin (r^2=0.03, p=0.0001) and a negative correlation between eGFR and UACR (r^2=0.27, p=0.0029). However, in some models both serum albumin and UACR were independent predictors of plasma CXCL16 when adjusted for eGFR.

A study of 146 CKD patients indicated that plasma CXCL16 levels were independently associated with eGFR (P<0.05) [189]. In 30 DKD patients showed that plasma CXCL16 levels were independently associated with eGFR and negatively correlated with blood albumin [190]. Plasma CXCL16 was positively correlated with 24-hour urine protein but negatively correlated with albumin in 50 active nephrotic syndrome patients [359]. The association between plasma CXCL16 and GFR may be related to

either a low clearance of CXCL16 when renal function deteriorates or to the presence of inflammation in CKD. In this regard, it is not known how CXCL16 is cleared from the circulation. The fact that CXCL16 correlated with proteinuria in nephrotic syndrome suggests that circulating CXCL16 is no excreted in great amounts in urine or that synthesis is higher than potential urinary losses.

The correlation between the plasma CXCL16 and serum alkaline phosphatase is of potential interest. Loss of GFR is associated with increase serum alkaline phosphatase which itself is a clinical sign associated with crude mortality and strong death risk [360]. A study included 313 normoalbuminuric type 1 DM patients showed that alkaline phosphatase levels were significantly associated with eGFR (p<0.007) [361]. The influence of eGFR on both plasma CXCL16 and serum alkaline phosphatase might explain the positive correlation found in our cohort between plasma CXCL16 and serum alkaline phosphatase. However, alkaline phosphatase and eGFR were independent predictors of plasma CXCL16 levels, and in our cohort there was no correlation between eGFR and alkaline phosphatase. Thus, an alternate explanation should be sought. Markers of systemic inflammation had been previously found to be associated with markers of CKD-MBD, including alkaline phosphatase levels in patients with CKD [173, 362-364]. Thus, our finding represented additional evidence supporting a link between systemic inflammation and CKD-MBD. In this regard, the trend towards the associations between plasma CXCL16 and serum phosphorus and $1,25(OH)_2D$ levels may be further evidence of the inflammation and CKD-MBD link.

In addition, loss of eGFR is associated with decreased phosphate clearance, high fibroblast growth factor-23 (FGF-23) and low soluble Klotho [365, 366]. A link of the association between CXCL16 and CKD-MBD parameters with these alterations may be hypothesized. Thus, inflammation decreases Klotho which has anti-inflammatory actions. High FGF-23 leads to decrease of serum $1,25(OH)_2D$ [366-368], which may suggest that the negative trend we found between plasma CXCL16 and serum $1,25(OH)_2D$ is related to loss of the eGFR.

Mean Pulse Wave Velocity (PWV)

Our results showed that 85 patients (44.5%) had PWV higher than normal for age with mean PWV 10.9±3.1 m/sec. Stepwise multiple regression analyses revealed that the independent determinants of PWV included non-modifiable (age, male gender, presence of DM) and modifiable factors (serum uric acid, UACR and resin calcium therapy for hyperkalemia).

These observations are concordant with previous studies that have also identified age, male gender, systolic BP, the presence of DM and UACR as determinants of higher PWV [310, 369-379]. Our observation that PWV increased to a greater extent with age in males vs. females is consistent with data from another study that identified male gender as an independent determinant of increased PWV in a large cohort of people with CKD [310, 371].

Increasing age was an independent predictor of PWV and arterial stiffness in most studies in healthy individuals [380] or CKD patients [371, 381]. In 1717 CKD stage 3 patients, age was the main predictor of PWV, while albuminuria was a weaker determinant and eGFR was not a determinant of PWV [371]. Even in ESRD on regular hemodialysis, age was an independent predictor of PWV and arterial stiffness [382]. Interestingly, some studies found that the carotid artery is more affected by the aging process than the femoral artery, even in healthy subjects. Local PWV and arterial distensibility have strong correlations with age at the carotid artery, but not with the femoral artery [383, 384]. The increase in arterial stiffness with age is proposed to be due to overproduction of abnormal collagen fibers and a loss of elastin from the extracellular matrix [385, 386]. It is not clear, however, whether this is a time dependant phenomenon directly related to chronological age or if it reflects exposure to other risk factors. Hypertension has long been recognized as a major determinant of arterial stiffness due to the associated medial hypertrophy [291]. The association between diabetes and arterial stiffness may be due to accumulation of AGE that provoke structural changes in the arterial wall [387] and the generation of reactive oxygen species that deactivate nitric oxide resulting in endothelial dysfunction [388].

Interestingly, UACR is independently associated with arterial stiffness assessed by PWV in subjects with DM and hypertension [379, 389, 390], as well as in a non-diabetic, non-hypertensive population [391], and the general population [19, 392, 393]. The association of albuminuria and vascular stiffness may represent the presence of endothelial injury leading to both phenomena [394], an effect of vascular stiffness on UACR or systemic consequences of albuminuria that may favor arterial stiffness. Functional animal studies are needed to answer this question. Meanwhile, renal doppler sonography calculation of the renal artery vascular resistance have demonstrated associations of a resistive index to proteinuria in CKD patients with and without DM [395], to albuminuria and also to a measure of aortic stiffness (brachial-ankle) in 150 patients with type 2 DM [396]. In patients with hypertension, a modest increase in renal resistive index was associated with a greater adjusted relative risk of albuminuria [397].

There is a strong association between hyperuricemia and CV morbidity or mortality [398-404]. In our study serum uric acid level was an independent determinant of PWV. Prior studies had linked serum uric acid with arterial stiffness in diabetes and non-CKD patients. In 106 male diabetic non-CKD patients [405] and in 3772 non-diabetic non-CKD individuals [406] serum uric acid was an independent determinant of PWV and arterial stiffness. However, for the first time we link serum uric acid and PWV in diabetic CKD patients. Our results may be related to the observation that use of allopurinol was independently associated with lower PWV in diabetic CKD patients, despite absence of significant correlation with serum uric acid levels [407].

Potassium chelation therapy was an independent determinant of PWV in our study. The significance of this observation is unclear, but merits further investigation. Thus the effects of potassium chelation therapy may be related to the chelation agent itself, which is known to release calcium in the gut and may favor phosphate binder-induced alkalosis [408]. Resin calcium was the potassium chelator in our study, composed of calcium polystyrene sulfonate [409-411]. Indeed, in hemodialysis patients, dialysate calcium and acute changes in the serum ionized calcium, even within physiological range, were associated with detectable changes of arterial stiffness and PWV [412]. Both calcium load and alkalosis may favor vascular calcification. However, patients on potassium chelation therapy had mean CO_2 of 26.1±3.0 mEq/l, while those patients who were not taking potassium chelation therapy had mean CO_2 of 28.1±3.4 mEq/l. Potassium chelation therapy may be a surrogate for hyperkalemia. Advanced CKD, DM, coronary artery disease, and peripheral vascular disease are independent predictors of hyperkalemia [413, 414]. Hyperkalemia is associated with increases in all-cause mortality and hospitalizations in those patients [415]. However, we did not encounter a significant correlation between the PWV and serum potassium, Furthermore, most of our patients were normokalemic. Conversely, potassium chelation therapy might be associated with intracellular potassium depletion. However, we do not have evidence pro or against this concept. Finally, the need for potassium chelation therapy may be a surrogate for medication increasing potassium levels or represent an underlying hormonal misbalance that impairs renal potassium excretion. In any case, there is some evidence for a relationship between potassium and arterial stiffness. Extracellular potassium has been reported to impact endothelial cell function and endothelial cell stiffness. An acute increase of potassium in the physiological range swells and softens the endothelial cell and increases the release of nitric oxide [416]. The relationship between endothelial stiffness and arterial stiffness is unclear. While a high-salt diet increases ambulatory arterial stiffness index, addition of potassium supplementation to the high-salt diet prevents the increase in ambulatory arterial stiffness index [417]. In a randomized, controlled trial a salt substitute (65% sodium chloride, 25% potassium chloride, 10% magnesium sulfate) over 12 months decreased BP and PWV compared with regular salt (100% sodium chloride) [418]. However, it is unclear what was driving the effect: low sodium or supplemental potassium or magnesium In this regard, in a randomized controlled trial increased potassium intake from fruit and vegetables or supplements did not modify PWV [419].

Delta upper limit of normal PWV (Delta PWV)

Delta PWV was calculated by subtracting the higher normal median PWV of healthy general population of the same age from the patient mean PWV (the difference between normal and abnormal PWV). All patients with positive values were considered to have an abnormally high PWV for their age, while those Within the normal limits were assigned a value of zero. All patients were included in the delta PWV statistical analysis.

Smoking, high systolic BP, and potassium chelation therapy were independent determinants of the high Delta PWV, while advanced age, increase urinary potassium and beta blocker therapy were independent determinants of low Delta PWV.

Previous studies showed that the systolic BP [369, 372, 375] and smoking were independent predictors of PWV in diabetic non-CKD patients [405] and in healthy individuals [420].

Our study disclosed that the potassium chelation therapy is an independent predictor of high delta PWV and low urinary potassium as an independent predictor of high delta PWV. This further information regarding the association of low urinary potassium does not add further light to the issue. Thus, low urinary potassium may represent whole body potassium depletion, an impaired ability to excrete urinary potassium, the consequence of the use of potassium chelators or the use of potassium sparing drugs. In this regard, the impact of potassium metabolism on arterial stiffness has been poorly documented in the literature.

A correlation between beta blockers and the delta PWV was observed. Beta blockers, especially vasodilating beta blockers, the most widely prescribed in our study, have been shown to decrease PWV in humans. The beta blocker nebivolol 5mg/day for 15 days in 13 essential hypertension non-diabetic, non-CKD patients significantly reduced central aortic pressure and PWV [421]. Nebivolol, but not atenolol,

acted directly on the arterial wall to increase arterial distensibility and was associated with reduced PWV [422, 423]. Nebivolol is a highly selective ϐ1-blocker currently on clinical use. Nebivolol enantiomers have different pharmacologic properties; D-isomer providing the beta-blocking activity and both the D- and L-isomers have an endothelial NO-dependent vasodilating effect [424, 425]. Thus nebivolol may impact vascular structure and function either directly by reducing BP or indirectly by increasing the bioavailability of NO [425]. Carvedilol; a vasodilating nonselective beta blocker, reduced PWV in hypertensive patients during 24 weeks of treatment [426]. However, no studies have addressed the effects of beta blockers on arterial stiffness using PWV in diabetic CKD patients.

Aortic Augmentation Index adjusted by heart rate (Aortic AIX@75)

Our study revealed that advanced age, high mean BP, female gender and low cardiac ejection fraction were independent predictors of high Aortic AIX@75. This was in concordant with previous studies performed in CKD and ESRD patients which showed that age, gender and mean arterial BP were independent predictors of Aortic AIX@75 [427-430]. Studies on in ESRD patients on hemodialysis revealed that low fractional shortening is independent predictor of Aortic AIX [318, 319]. The only factor associated to Aortic AIX@75 in our study that is modifiable is BP.

Subendocardial viability ratio (SEVR; or Buckberg Index)

The mean±SD of the SEVR were 141.1±30.0 %. This high value means a good myocardial perfusion. The SEVR reflects myocardial oxygen supply and demand, with low values representing a lesser degree of myocardial perfusion [431]. The ratio of diastolic time index (pressure load during diastole) vs. tension time index (pressure load during systole), that is, the integral of pressure and time during diastole and systole, respectively, has been shown to correlate well with the ratio of subepicardial to subendocardial blood flow, and therefore represents an index of subendocardial viability [432]. The SEVR is an independent predictor of coronary flow reserve in patients with essential hypertension [433]. The myocardial oxygen demand slightly increases in relation to blood supply in untreated hypertensive patients [434].

Independent predictors of low SEVR (representing worse myocardial perfusion) were female gender, high pulse pressure, high mean PWV and proton pump inhibitor therapy, while advanced age, high 25(OH)D and beta blocker therapy were independent predictors of high SEVR.

Our univariate analysis did not show a significant correlation between age and SEVR, however we included age in the multivariate regression analysis because of its relationship with arterial stiffness and cardiac performance. Low SEVR has been reported in several studies to be independently associated with old age [318, 427, 435, 436]. High SEVR was independently associated with old age in some multivariate models explaining about 30 % of the variability of SEVR. However, this was not the case in other models.

The association of low SEVR with female gender had been previously described in type 1 DM [435] and healthy population [318], however the association with low serum 25(OH)D [437] had been previously described in healthy population.

The observed positive correlation between beta blocker therapy and SEVR might have been related to the BP control. There were no significant differences in mean diastolic BP between patients on beta blocker therapy and those not on beta blockers, while systolic BP and pulse pressure were significantly higher in patients on beta blocker therapy (146.4±20.2 mmHg vs. 139.6±18.1 mmHg and 70.2±18.7 mmHg vs. 60.1±15.5 mmHg, respectively) . However, beta blocker use was an independent predictor of SEVR when pulse pressure was on the same multivariate model and the fact that patients on beta blockers therapy had higher pulse pressure does not fit with the inverse association between SEVR and pulse pressure. The inverse association between SEVR and pulse pressure had been previously reported. SEVR tended to be lower in hypertensive patients with pulse pressure > 60 mmHg than in hypertensive patients with normal pulse pressure [438]. In a meta-analysis pulse pressure but not the mean BP was an independent predictor of CV complications and all-cause mortality in older hypertensive patients [439]. Pulse pressure is generated by the left ventricle during systole and is dampened by the compliance of the aorta. Loss of aortic compliance with age or CVD will lead to greater pulse pressure. In addition, hypertension increases the LV afterload thus increases LV workload and consequently myocardial oxygen demand, increasing the susceptibility for myocardial ischemia [432].

In this cohort, PWV was an independent predictor of SEVR. In 54 non-diabetic hemodialysis patients severe vascular calcification was associated with higher PWV and lower SEVR. Ultrafiltration

improved both PWV (mean reduction of 16%) and SEVR (increase of 13%) and the severity of vascular calcifications influenced the effect of ultrafiltration on these two parameters [440].

In our study use of proton pump inhibitors was associated with low SEVR. This finding is concordant with the result of a study of 387 ESRD hemodialysis patients which reported that long-term treatment with proton pump inhibitors, especially in the presence of warfarin treatment, was associated with vascular calcification [441]. Another study found that proton pump inhibitors use was associated with increased major adverse CV events in patients with unstable coronary syndromes [442]. Proton pump inhibitors inhibit the activity of DDAH, an enzyme necessary for CV health. DDAH metabolizes ADMA; a uremic toxin which is an endogenous and competitive inhibitor of NOS. By inhibiting endothelial NOS, ADMA may increase CV risk [442, 443].

CONCLUSIONS

The present thesis allows to draw the following conclusions:

1. Plasma PCSK9 values in diabetic CKD patients are very variable and the factors underlying this variability are unclear. The use of lipid lowering therapies containing fibrates is associated with higher plasma PCSK9. However, PCSK9 did not correlate with features of vascular injury.

2. Plasma CXCL16 values in diabetic CKD patients increased with renal function deterioration and albuminuria and correlate with parameters of bone mineral metabolism, but not with features of vascular injury.

3. Arterial stiffness was very prevalent in CKD patients. Among modifiable factors associated with arterial stiffness we found serum uric acid, albuminuria and potassium metabolism.

CONCLUSIONES

La presente tesis permite extraer las siguientes conclusiones:

1. Los valores de PCSK9 plasmáticos en pacientes con ERC diabéticos son muy variables y los factores que subyacen a esta variabilidad no están claros. El uso de terapia hipolipemiante con fibratos se asocia con niveles de PCSK9 más altos. Sin embargo, PCSK9 no se relacionó con patología cardiovascular.

2. Los valores de CXCL16 plasmáticos en pacientes diabéticos con ERC aumentan con el deterioro de la función renal y la albuminuria, y se correlacionaron con parámetros del metabolismo mineral óseo, pero no con patología cardiovascular.

3. La rigidez arterial es muy frecuente en los pacientes con ERC. Entre los factores modificables asociados con la rigidez arterial se encuentran la uricemia, albuminuria y el metabolismo de potasio.

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ANNEX I

Biomarkers characteristics of healthy individuals

	Age (years)	Gender	PCSK9 (ng/ml)	CXCL16 (ng/ml)
Control 1	31	Male	204	3.19
Control 2	50	Male	221	3.33
Control 3	32	Male	248	3.27
Control 4	34	Female	372	2.65
Mean ± SD	36.8±8.9	----------	261.2±76.2	3.1±0.3

Biomarkers characteristics of non diabetic CKD patients

	Age (years)	Gender	PCSK9 (ng/ml)	CXCL16 (ng/ml)	eGFR (ml/min/ $1.73m^2$)	UACR (mg/g)
CKD 1	72	Female	230	5.33	28	1189.9
CKD2	64	Male	352	5.87	12	430.9
CKD 3	79	Male	441	4.61	35	492.3
Mean ± SD	71.0±8.5	---------	341.0±105.9	5.3±0.6	25.0±11.8	704.4±421.6

ANNEX II

General Description of non diabetic CKD patients (with biomarkers)

Variable		Value
Total number of patients n (%)		3 (100)
DM n (%)		0
Hypertension n (%)		2 (66.7)
CVD		1 (33.3)
CKD Etiololgy n (%)	Vascular	1 (33.3)
	Chronic interstitial nephritis	2 (66.7)
CKD Stages n (%)	Stage 3B	1 (33.3)
	Stage 4	1 (33.3)
	Stage 5	1 (33.3)
Cigarette smoking n (%)	Non smoker	2 (66.7)
	Active smoker	1 (33.3)
	Ex-smoker	0
Alcohol consumption n (%)		0
Age (years)		71.0±8.5
Gender n (%)	Males	2 (66.7)
	Females	1 (33.3)
Height (cm)		159.5±4.9
Weight (kg)		69.3±3.1
Body Mass Index (kg/m^2)		28.2±2.6
Systolic Blood Pressure (mmHg)		143.3±12.3
Diastolic Blood Pressure (mmHg)		81.3± 20.2
Mean Blood Pressure (mmHg)		102.7±17.4
Pulse Pressure (mmHg)		62.0±14.7

Medications of the non diabetic CKD patients (with biomarkers)

Medication	Number of patients (%)
Total patients	3 (100)
Paricalcitol (mcg/w)	3 (100)
Calcifediol (IU/w)	3 (100)
Calcitriol (mcg/w)	0
Cinacalcet (mg/w)	1 (33.3)
Lantanum	1 (33.3)
Sevelamer	2 (66.7)
Aluminium- based phosphate binders	0
Calcium-based phosphate binders	0
Calcium Supplement	0
Calcium Polystyrene Sulfonate (Potassium chelator)	2 (66.7)
Iron supplement	2 (66.7)
ESAs	1 (33.3)
Oral hypoglycemic agents	1 (33.3)
Insulin	0
Statin	2 (66.7)
Fibrate	0
Ezetimibe	0
Omega 3-Fatty Acids	0
ACEIs	1 (33.3)
ARBs	0
Spironolacatone	0
Calcium Channel Blockers	2 (66.7)
Beta Blockers	1 (33.3)
Alpha Blockers	0
Alpha & Beta Blockers	0
Diuretics	0
Proton Pump Inhibitors	2 (66.7)
Antiplatelets	0

Dosage for selected medications of non diabetic CKD patients (with biomarkers), Only patients received the medications were analyzed

Medication	Mean±SD
Paricalcitol (mcg/w)	5.0±2.6
Calcifediol (UI/w)	1680.0±415.7

Serum analysis parameters of non diabetic CKD patients (with biomarkers)

Laboratory Parameters / (n. of patients)	Non diabetic CKD control
Total patients n (%)	3 (100)
Serum Creatinine (mg/dl) / (3)	3.1±1.9
GFR (MDRD) (ml/min/1.73 m^2) / (3)	25.0±11.8
Serum Glucose (mg/dl) / (3)	105.7±29.9
Serum Uric Acid (mg/dl) / (3)	6.6±1.3
Serum HbA1C (%) / (3)	6.0±0.6
Haemoglobin (g/dl) / (3)	13.2±2.4
Serum Albumin (g/dl) / (3)	3.9±0.6
Serum hsCRP (mg/dl) / (2)	0.925±0.300
Serum Prealbumin (mg/dl) / (2)	25.8±2.1
Serum Transferrin (mg/dl) / (3)	252.7±51.7
Serum Ferritin (ng/ml) / (3)	84.7±80.3
Serum Total Cholesterol (mg/dl) / (3)	157.3±18.7
Serum LDL Cholesterol (mg/dl) / (3)	71.0±9.6
Serum HDL Cholesterol (mg/dl) / (3)	62.0±7.5
Serum Triglycerides (mg/dl) / (3)	122.0±41.9
Plasma Renin (ng/ml/hr) / (2)	31.55±18.90
Plasma Aldosterone (pg/ml) / (2)	376.0±4.2
Serum CO_2 (mEq/L) / (3)	22.7±6.1
Serum LDH (IU/l) / (3)	438.7±39.6
Serum Sodium (mmol/l) / (3)	141.3±2.5
Serum Potassium (mmol/l) / (3)	4.7±0.3
Serum Magnesium (mg/dl) / (3)	2.1±0.3
Serum Calcium (mg/dl) / (3)	9.4±0.3
Serum Phosphorus (mg/dl) / (3)	3.90± 0.06
Serum Alkaline Phosphatase (IU/l) / (3)	112.3±36.5
Serum Iron (ε g/dl) / (3)	70±42
Serum TIBC (ε g/dl) / (3)	320.7±65.5
Serum intact PTH (pg/ml) / (3)	166.8±60.8
Serum 25(OH)D (ng/ml) / (3)	31.4±4.8
Serum 1,25$(OH)_2$D (pg/ml) / (2)	23.0±1.4
Serum Vitamin B12 (pg/ml) / (2)	504.0±11.3
Serum Folic Acid (ng/ml) / (2)	6.1±2.1
Serum TSH (ε IU/ml) / (3)	2.7±1.3
Serum Free T3 (pg/ml) / (3)	3.3±0.5
Serum Free T4 (ng/dl) / (3)	1±0
SerumVitamin E (mg/l) / (2)	0.685±0.200
SerumVitamin E (ε g/ml) / (2)	17.8±7.4
Plasma PCSK9 (ng/ml) / (3)	341.0±105.9
Plasma CXCL16 (ng/ml) / (3)	5.27±0.60

Urinalysis parameters of non diabetic CKD patients (with biomarkers)

Laboratory Parameters / (n. of patients)	Non diabetic CKD control
Total patients n (%)	3 (100)
Creatinine Clearance (ml/min) / (3)	19.3±5.9
Diuresis (ml/24h) / (3)	2866.1±950.4
Total Proteinuria (mg/24h) / (3)	2318 (1577, 3537)
Glycosuria (mg/dl) / (3)	0
Spot Proteinuria (mg/dl) / (3)	61.0 (52.5,112.5)
UPCR (mg/g) / (3)	2006.5 (1567.4, 6193.2)
Total Microalbuminuria (mg/24h) / (3)	154.7 (25.0, 326.1)
UACR (mg/g) / (3)	95.4 (18.0, 273.7)
Spot Creatinuria (mg/dl) / (3)	28.4±11.7
Urinary Sodium (mmol/L) / (3)	73.7±25.1
Urinary Potassium (mmol/L) / (3)	18.7±11.0
Urinary Magnesium (mg/dl) / (3)	2.4±1.0
Urinary Magnesium (mg/24h) / (3)	63.9±20.1
Urinary Magnesium (mg/g Cr.) / (3)	0.085±0.010
FEMg (%)/ (3)	17.0±7.2
Urinary Calcium (mg/dl) / (3)	3.1±1.2
Phosphaturia (mg/dl) / (3)	21.7±6.6
Phosphaturia (mg/24h) / (3)	526.0 (474.7, 698.1)
Phosphaturia (mg/mg Cr.) / (3)	0.796±0.100

Distribution of UACR among the non diabetic CKD patients (with biomarkers)

UACR (mg/g)	Non diabetic CKD control group
	Number of patients (%)
<30	0
30-299	0
300-1000	2 (66.7%)
>1000	1 (33.3%)
Total	3 (100)

Echocardiograms of non diabetic CKD patients (with biomarkers)

Variable	Value
Total number of patients n (%)	3 (100)
Ejection Fraction (%)	60±0
Left Ventricular Diameter (mm)	44.3±5.0
Interventricular septum (mm)	11.2±1.0
Left Ventricular Hypertrophy n (%)	2 (66.7)
Alterered Relaxation n (%)	3 (100)
Cardiomyopathy n (%)	0
Valvular Calcification n (%)	1 (33.3)
Pericardial Effusion n (%)	0

ANNEX 3

Publications

1: González-Parra E, Herrero JA, **Elewa U**, Bosch RJ, Arduán AO, Egido J. Bisphenol a in chronic kidney disease. Int J Nephrol. 2013;2013:437857. PubMed PMID: 23997953;

2: **Elewa U**, Sanchez-Niño MD, Martin-Cleary C, Fernandez-Fernandez B, Egido J, Ortiz A. Cardiovascular risk biomarkers in CKD: the inflammation link and the road less traveled. Int Urol Nephrol. 2012 Dec;44(6):1731-44. PubMed PMID: 22965378.

3: Fernández Fernández B, **Elewa U**, Sánchez-Niño MD, Rojas-Rivera JE, Martin-Cleary C, Egido J, Ortiz A. 2012 update on diabetic kidney disease: the expanding spectrum, novel pathogenic insights and recent clinical trials. Minerva Med. 2012 Aug;103(4):219-34. PubMed PMID: 22805616.

4: Nastou D, Fernández Fernández B, **Elewa U**, González-Espinoza L, González-Parra E, Sánchez-Niño MD, Ortiz A. Next generation phosphate binders: focus on iron-based binders Drugs. Accepted 2014

Abstracts Accepted To Congresses

1: **Elewa U**, Fernandez B, Clary-Martin C, Egido J, Ortiz A, Gonzalez-Para E
Evaluation of Cardiovascular Risk in Chronic Hemodialysis Patients with the Pulse Wave Velocity
XLII Congreso Nacional de la Sociedad Española de Nefrología - VII Congreso Iberoamericano, Gran Canaria, Spain, 6th -9th October 2012

2: **Elewa U**, Fernandez B, Tabikh A, Egido J, Ortiz A
Serum 25-Hydroxyvitamin D Inversely Correlates With Pulse Wave Velocity (PWV) In Non-Dialysis Diabetic Chronic Kidney Disease (CKD) Patients
International Society of Nephrology - World Congress of Nephrology 2013, Hong Kong, 31st May ï 4th June 2013

3: Martin-Cleary C, Fernandez B, **Elewa U**, Mahillo I, Egido J, Ortiz A
Classical Monocytes Express High Levels of CXCL16 in Hemodialysis Patients
1st Symposium on Biomedical Research, Universidad Autónoma de Madrid, Madrid, 21st March 2014

4: **Usama Elewa**, Waled Bichari and Khaled Aboseif
Assessment of peripheral vascular disease in hemodialysis Patients using Toe Brachial Index
ERA EDTA 51st congress, Amsterdam, Netherlands, 31st May ï 3rd June 2014

5: **Elewa U,** Nastou D, Fernandez B, Mahillo I, Egido J, Ortiz A
Prevalence, causes and risk factor for hypomagnesaemia in non-dialysis CKD patients
ERA EDTA 51st congress, Amsterdam, Netherlands, 31st May ï 3rd June 2014

6: **Elewa U,** Alegre R, Fernandez B, Mahillo I, Egido J, Ortiz A
Modifiable predictors of vascular stiffness
ERA EDTA 51st congress, Amsterdam, Netherlands, 31st May ï 3rd June 2014